Solid Phase Peptide Synthesis

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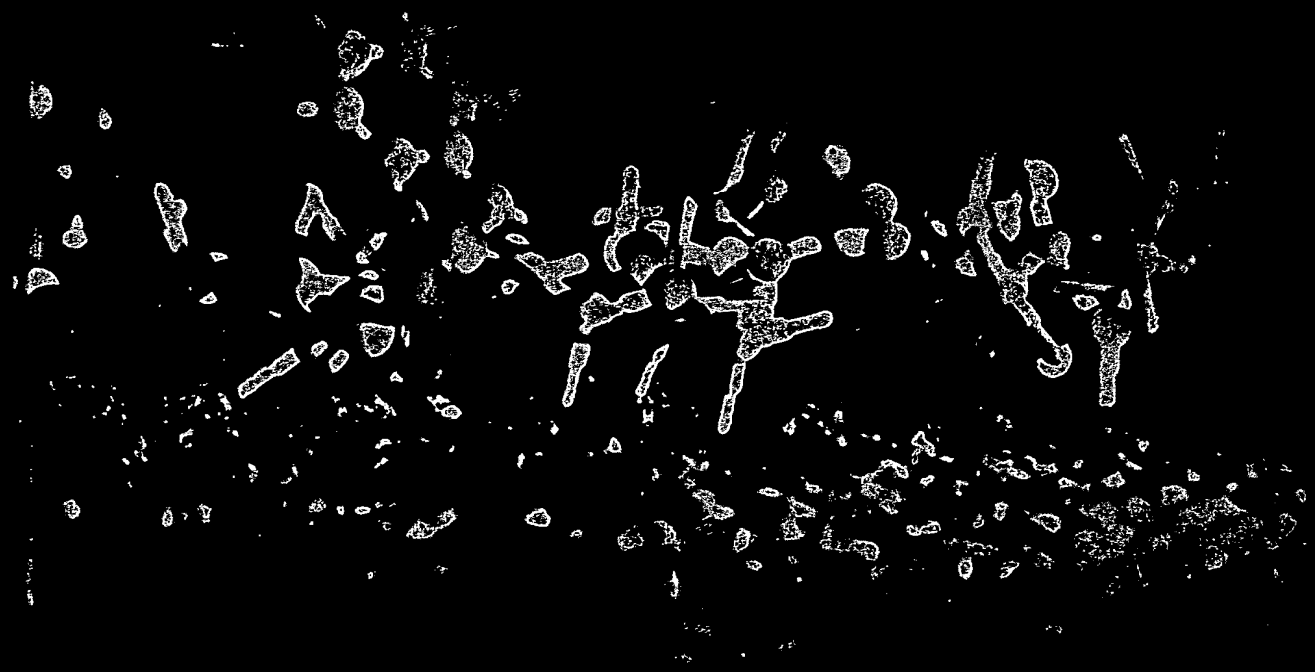
Second Edition



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The Chemistry of Solid Phase Peptide Synthesis

A. INTRODUCTION: THE SOLID PHASE REACTION PRINCIPLE

Solid phase peptide synthesis was introduced by Bruce Merrifield in 1963 in an effort to overcome many of the problems of peptide synthesis in solution as it was practiced at that time. SPPS was the first practical application of the use of insoluble, polymer-bound reagents in organic synthesis. Of the several possible approaches for polymer-assisted peptide synthesis, Merrifield chose to attach the C-terminal residue of the peptide to be synthesized to an insoluble polymer and grow the peptide chain toward the amino end of the peptide. The publication of more than 1,000 papers on SPPS attests to the need for SPPS and the wisdom of Merrifield's choice of method. Several reviews have appeared, the most recent being the comprehensive and authoritative one of Barany and Merrifield;[1] that review is an essential reference for all practitioners of SPPS. In this chapter we do not attempt to present another exhaustive coverage of SPPS chemistry, but rather present a selective discussion of the chemistry most needed and useful for practical SPPS. Continuing the policy of our earlier book,[2] we do not present here a discussion of the general principles and practices of peptide synthesis. We assume, rather, that the reader is already familiar with these principles. For general information on peptide synthesis the reader is referred to the book of Bodanszky *et al.*[3], the review of Finn and Hofmann,[4] and the encyclopedic work of Wünsch.[5]

The fundamental premise of solid phase synthesis is that amino acids can be assembled into a peptide of any desired sequence while one end of the chain is anchored to an insoluble support. As mentioned above, in practical SPPS the carboxyl terminus of the peptide is linked to the polymer. After the desired sequence of amino acids has been linked together on the support, a reagent can be applied to cleave the chain from the support and liberate the finished peptide into solution. All the reactions involved in the synthesis should be carried 100% to completion, so that a homogeneous product could be obtained. The great advantage of using a polymer-supported peptide chain is that all the laborious purification at intermediate steps in the synthesis is eliminated, and simple washing and filtration of the peptide-resin is substituted. By use of a suitably designed reaction vessel, all the synthesis can be carried out in one container without any transfer of material from one vessel to another. Thus mechanical loss of material during transfer is eliminated as well as the losses associated with purification of intermediates during solution phase synthesis.

This basic idea of SPPS is illustrated in Figure 1-1. The insoluble support is a synthetic

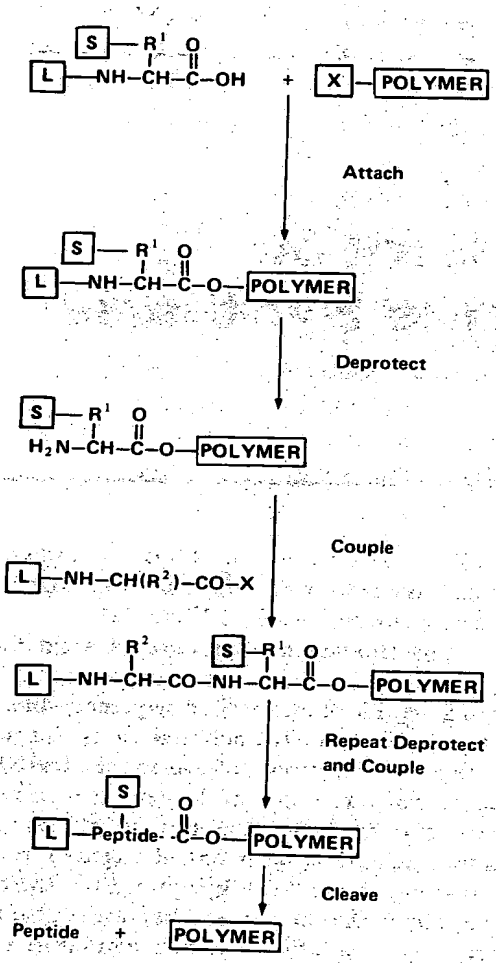


FIGURE 1-1.
The Scheme of Solid Phase Peptide Synthesis.

polymer which bears reactive groups (X). The amino acid which will form the C-terminal residue of the peptide to be synthesized is converted to a derivative in which its amino group is protected by a labile protecting group (L). Using appropriate chemistry, this derivative of the C-terminal amino acid is coupled to the reactive polymer. At this point the repetitive cyclic part of SPPS begins. A reagent is applied to the protected aminoacyl polymer to remove the labile blocking group (L) from the amino acid residue. This reagent must not harm the link of the C-terminal residue to the polymer in any way. Moreover, if the amino acid attached to the polymer (and all amino acids in the peptide to be synthesized) contains a side-chain reactive functional group, that functional group must be blocked by a stable blocking group (S) which will remain completely intact throughout the synthesis, but which can be removed finally to yield the free peptide. Following removal of the labile protecting group, the next amino acid is coupled to the aminoacyl polymer by use of a suitable coupling reaction. Again, the α-amino group must be protected with the labile protecting group. This cycle of deprotection and coupling is then repeated with each amino acid which is to be incorporated into the peptide chain. Finally, after the entire blocked peptide has been assembled on the polymer support, a different type of reagent is applied to cleave the peptide from the polymer and allow it to be dissolved. The blocking groups which have protected side-chain functional groups must also be removed, and usually are chosen so that they can be removed simultaneously with cleavage of the peptide from the resin.

The critical requirements for synthesis of homogeneous peptides by SPPS are that the deprotection and coupling reactions go fully to completion at every step of the synthesis and that the side-chain blocking groups and the peptide-resin bond be completely stable throughout all these steps. In practice, the deprotection reagent must be much more vigorous than is usually necessary to remove the same labile protecting group from amino acids in solution, since not only must the last trace of protecting group be removed, but in many cases protected peptides attached to a polymer matrix may be significantly more difficult to deprotect than are single amino acids in solution. Likewise, the coupling reaction must be driven to completion by use of a large excess of the activated amino acid being attached to the peptide-resin at each step of the synthesis. Several peptide chemists have amused themselves by making calculations which demonstrate that SPPS is impossible. They have pointed out with delight that if each step in the synthesis should proceed only 95%, or 98%, or 99% to completion, very soon the undesirable

side-reaction products would account for the majority of the material on the resin. These side-products would presumably be principally shorter peptides lacking one or more residues of the desired sequence, due to failure of deprotection or coupling reactions to proceed to completion. Such shorter peptides, commonly called "deletion peptides" or "deletion sequences" do, in fact, occur in SPPS, but experience has shown that by far the majority of steps in SPPS can be made to go quantitatively to completion.

A great advantage of SPPS is its speed. With the "classical" system of Merrifield SPPS, peptide chains are usually assembled at the rate of one residue per 4 hours. Manual SPPS allows convenient elongation of peptide chains at the rate of two or three residues per day, and automatic synthesizers currently available can carry out synthesis at the rate of six or more residues per 24 hours. This speed and simplicity of SPPS has made it practical for individual peptide chemists to embark upon programs of analog synthesis which would be impractical using solution methods. The many thousands of peptides synthesized by SPPS in the two decades since it was introduced attest to the fact that it is indeed practical and that it can be carried out successfully. It is true, however, that most of the peptides synthesized by SPPS have been relatively small ones, usually containing less than 20 amino acids. Synthesis of such peptides is often routine and straightforward without significant complications. If that were uniformly the case, there would be little remaining challenge in peptide synthesis. However, peptides frequently demonstrate properties unanticipated from consideration of the properties of the individual constituent amino acids due to conformational or steric characteristics of the assembled peptide. In addition, when the blocked peptide is being assembled upon and within a polymer matrix, additional interactions occur which cannot be predicted from any standard investigations of reaction mechanisms or kinetics. Thus, small peptides can occasionally give rise to serious problems in synthesis, and problems can be expected in the synthesis of long peptides by SPPS. On the other hand, synthesis of many peptides proceeds in a completely routine fashion.

1. Merrifield Solid Phase Synthesis

The system now considered standard for SPPS of small or medium-sized peptides is given in Figure 1-2. This system uses as resin support a 1% cross-linked polystyrene (copolymer of styrene with 1% divinylbenzene), which swells extensively in the solvents used for the synthesis (dichloromethane, dimethylformamide). This swelling allows rapid penetration of solvents and reagents into the resin beads so that efficient reactions may take place. The polystyrene resin beads are functionalized by chloromethylation, which introduces benzyl chloride-type groups into the polymer. These halogens are reactive, and when treated with the salt of a protected amino acid will form an ester, linking the protected amino acid covalently to the resin as a substituted benzyl ester.

The standard protecting group for α-amino functions is the Boc group. This group is removed by treatment with rather dilute solutions of strong acids such as 25% TFA in DCM. Earlier work used solutions of anhydrous HCl in dioxane. The newly exposed amino group is present as the acid salt. It is then converted to the free base by treatment with a tertiary amine, usually triethyl amine in DCM. The next Boc-amino acid is then coupled to the aminoacyl resin, usually by use of DCC. Less frequently, preformed Boc-amino acid active esters or symmetrical anhydrides may be used in the coupling step. Following completion of assembly of the desired blocked peptide on the resin, the peptide-resin is treated with anhydrous HF to cleave the benzyl ester linking the peptide to the resin in order to liberate the free peptide. Side-chain functional groups of amino acids are usually blocked during the synthesis by benzyl-derived blocking groups, which are also cleaved from the peptide simultaneously with its removal from the polymer support. The free peptide is then extracted from the resin with a suitable solvent, purified, and characterized.

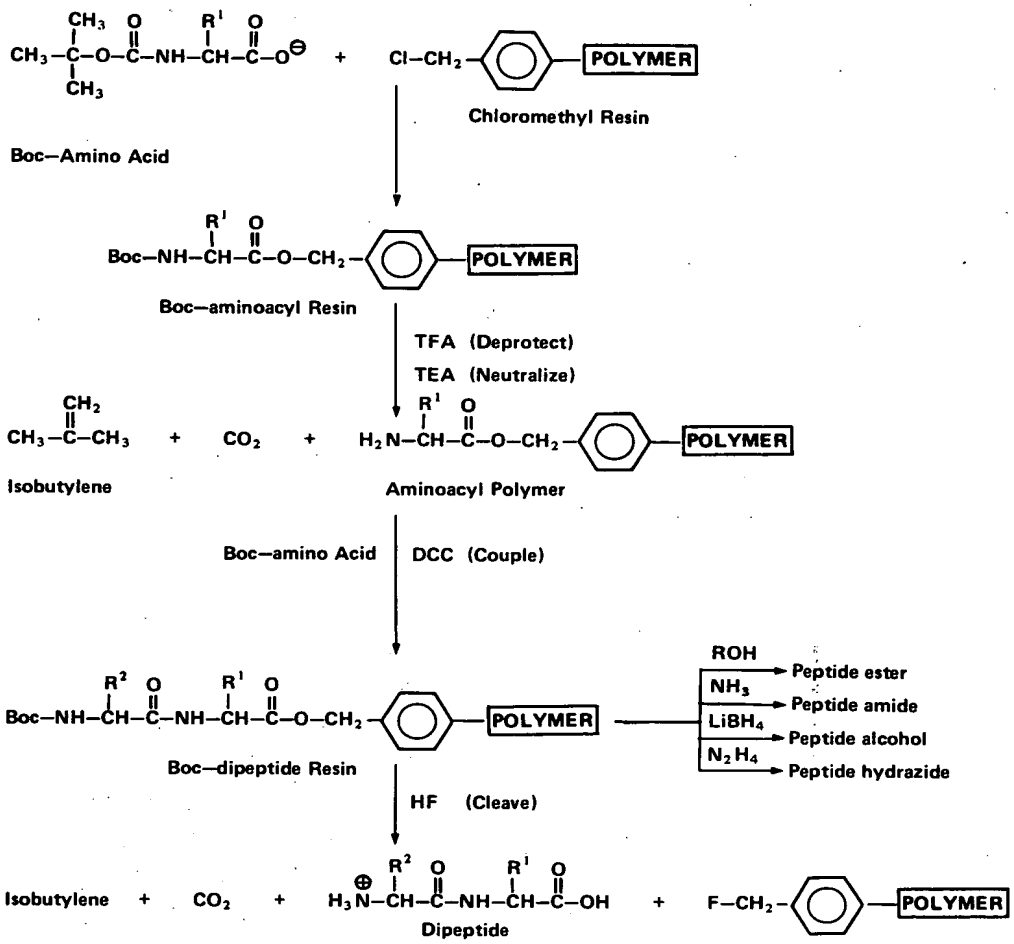


FIGURE 1-2.
The "Classical" Merrifield Scheme of Solid Phase Peptide Synthesis.

2. *Strategy in SPPS*

Although the classical system of Merrifield SPPS described in the preceding section has been most used for synthesis, other combinations are available for special purposes. Several sets of combinations of resin protecting groups, blocking groups, and deprotection and cleavage reagents are given in Table 1-1, along with the purpose for which they have been used. While the classical system can be expected to yield satisfactory results for synthesis of most small peptides, for larger peptides and for special purposes the choice of the proper combination of these factors can make the difference between success and failure of the project.

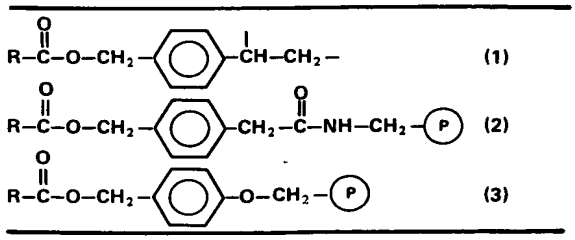
The problem of inappropriate relative reaction rates arises when the classical system is applied to synthesis of long peptides. Since acidolysis is used both for removal of the Boc groups during each step of the synthesis as well as cleavage of the side-chain blocking groups and cleavage of the peptide from the resin at the end of the synthesis, successful SPPS requires a large difference in reactivity of these groups. The ester link of the C-terminal amino acid to the resin is not absolutely stable under the conditions necessary to assure complete removal of Boc groups; a small amount of peptide is lost from the resin at each step of the synthesis. Loss of peptide may be as much as 1% per cycle in some cases. While this

Table 1-1. The Strategy of Solid Phase Peptide Synthesis

SPPS SYSTEM USED	PEPTIDE-RESIN LINK	ALPHA PROTECTION	DEPROTECTION REAGENT	SIDE-CHAIN BLOCKING	CLEAVAGE REAGENT
Classical	Benzyl ester	Boc	TFA, HCl	Benzyl	HF, HBr
Stable (long chain)	Pam	Boc	TFA, HCl	Benzyl	HF, HBr
	Benzyl ester	Bpoc	Dilute TFA	Benzyl	HF
	Benzyl ester	Bpoc	Dilute TFA	*t*-Butyl	HF
Labile	Ether resin	Bpoc	Dilute TFA	*t*-Butyl	TFA
Orthogonal	Ether resin	Fmoc	Piperidine	*t*-Butyl	TFA
Segment synth.	Ether resin	Fmoc	Piperidine	Benzyl	TFA
	t-Butyl resin	Fmoc	Piperidine	Benzyl	TFA
	Hydrazide resin	Fmoc	Piperidine	Benzyl	TFA
Segment assembly	Benzyl ester	Fmoc	Piperidine	Benzyl	HF
Peptide amides	MBHA, BHA	Boc	TFA, HCl	Benzyl	HF
Peptide alcohols	Benzyl ester	Boc	TFA, HCl	Benzyl	$LiBH_4$

may not present serious difficulty for synthesis of short peptides, such loss is serious when synthesis of long peptides will be attempted. Not only does this loss lead to decreased yield of final product, but it gives rise to termination of the peptide chain by internal acylation (see Sections I and K, below). This difficulty can be overcome by use of either a more labile α-blocking group for the amino acids or a more stable peptide-resin link. Both these approaches have been used. The classical peptide-resin link can be used with Bpoc, Ddz or Poc amino acids, using more dilute TFA for deprotection at each step of the synthesis. On the other hand, Boc-amino acids can be used with a more stable peptide-resin link, such as that provided by the Pam resin introduced by Merrifield. The principal factor which determines lability to acidolysis of a peptide ester or amide is the electron density at the carbonyl group. High electron density facilitates attack of a proton on the ether oxygen of the ester (see Figure 1-3). In the classical Merrifield SPPS resin (**1**) the link is slightly more labile than a standard benzyl ester due to the electron-donating ability of the alkyl chain of the polystyrene resin. In the Pam resin, (**2**) the electron-withdrawing power of the acetic acid amide group on the phenylene ring to which the peptide is attached provides additional stability and makes peptides linked to the Pam resin about 100 times as stable to acidolysis as those on the classical resin.

Not all peptides are stable under the conditions of classical SPPS. In cases where anhydrous HF is harmful, a much more labile system may be used for the synthesis. Such a system is provided by the ether resin, (**3**) used with Bpoc, Fmoc, or Nps amino acids.



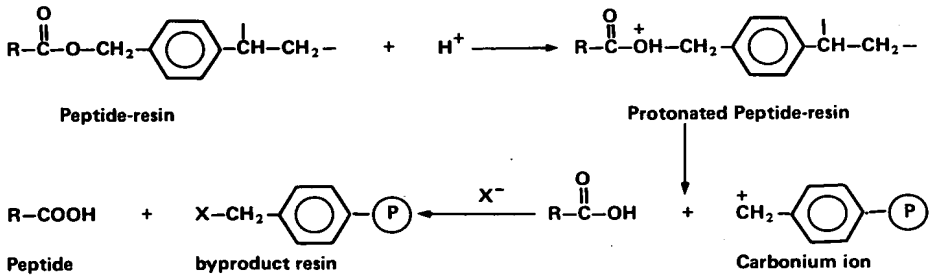


FIGURE 1-3.
The Mechanism of Acidolytic Ester Cleavage.

The standard approach in SPPS involves addition of Boc amino acids, one at a time, to an aminoacyl resin. If any deprotection or coupling step fails to go absolutely to completion, the product can be expected to contain "deletion peptides", which lack one or more amino acids of the desired sequence. While such deletion sequences may be separated from the desired product in many small peptides, the problem presented by removal of such unwanted by-products from large peptides is clearly very serious. Normal peptide purification procedures cannot be expected to remove such contaminants unequivocally. If, on the other hand, small blocked peptides are coupled to the resin instead of individual amino acids, the deletion sequences will differ from the desired sequence by more than a single amino acid residue and their removal would presumably be simpler. Such segment synthesis in SPPS entails much more work than the classical single amino acid coupling procedures. Suitable blocked peptides must be synthesized separately and then incorporated into the desired sequence. Segment synthesis has been used with good success in a few cases.[6,7] Segment synthesis has also been used as a means to overcome "impossible" coupling problems in synthesis of certain long peptides by the stepwise method. For example, extensive termination of peptide chains has been seen in certain sequences at glutamine and proline residues. In those cases the problems may often be overcome by synthesis of a dipeptide containing the difficult sequence and coupling the dipeptide to the peptide-resin. Racemization is a serious problem in segment synthesis (see section K.5, below).

Another choice of strategy is involved in the synthesis of peptide amides. These derivatives are extremely important, since many naturally-occurring peptide hormones are present as the amide. Many peptide amides have been synthesized by ammonolysis of classical Merrifield peptide-resins (see Figure 1-2). This is a practical procedure when the peptide does not contain aspartic or glutamic acid residues. When the latter are present as esters, ammonolysis will convert these residues to glutamine and asparagine. As a matter of fact, this approach has been occasionally used for synthesis of glutamine and asparagine-containing peptides. If glutamic and aspartic acids are not present, this approach also offers the possibility of obtaining both a free peptide and a peptide amide from a single synthetic run.

Aside from problems with glutamic and aspartic acids, ammonolysis reactions are slow and frequently give poor yields of products. Furthermore, if the peptide contains blocked side-chain functional groups, these must be subsequently removed by cleavage with HF or other suitable reagents. These problems have been largely eliminated by the development of solid-phase resins which yield peptide amides directly upon cleavage with anhydrous HF. These benzhydrylamine and methylbenzhydrylamine resins are discussed in greater detail in section C, below.

3. *Orthogonal Systems*

In the procedures discussed above, the selectivity of removal of the protecting group at each step of the synthesis and of the side-chain blocking groups (and cleavage of the peptide-resin link) at the end of the synthesis depends upon differences in rate of acidolytic cleavage of these two categories of groups. Problems attendant upon the use of such systems have been discussed above. To overcome these difficulties, orthogonal systems of SPPS have been developed. In these systems the side-chain blocking groups and the peptide-resin link are completely stable to the reagent used to remove the α-protecting group at each step of the synthesis. The earliest approach to orthogonal SPPS involved use of Nps-amino acids; the Nps group can be removed by thiolysis, which does not attack the side-chain groups or the peptide-resin link. Instability and lack of commercial availabilty of the Nps-amino acids have inhibited development of this system. More attention recently has been given to the use of Fmoc-amino acids; the Fmoc group is removed by treatment with a secondary amine. Side-chain functions are usually blocked by *t*-butyl derived blocking groups and the resin is usually the ether resin developed by Wang. This ether resin, while much more labile than the standard Merrifield resin to acidolysis, is much more stable to aminolysis. Indeed, the Merrifield resin is not suitable for use with Fmoc amino acids. Although some impressive solid phase synthesis has been done using Fmoc-amino acids, the high prices of the derivatives and lack of commercial availability of the complete set of derivatives make this approach less attractive if satisfactory results can be obtained by the more conventional procedures. A recent addition to the category of orthogonal systems is the dithiasuccinyl blocking group introduced by Barany and Merrifield.[8]

4. *Materials for SPPS*

Commercial availability at reasonable prices of all the materials needed for the classical system of SPPS makes this system very attractive. Recent years have seen great advances in availability and purity of amino acid derivatives. Chloromethyl resin for routine peptide synthesis and BHA resin for peptide amide synthesis are available from several sources. For the investigator who does very little peptide synthesis, resins already containing the first Boc-amino acid attached to them are available commercially.

It cannot be assumed that commercially available materials for SPPS will always be of acceptable purity; constant vigilance is necessary. All amino acid derivatives should be checked for purity by TLC and melting point; tables of derivatives are given in the Appendix for reference.

While optical purity of L-amino acid derivatives is not generally a problem now, the same cannot be said for D-amino acid derivatives. These latter derivatives should always be checked for optical rotation. While polarimetry is usually not sufficiently accurate to indicate the presence of small amounts of L-amino acid, at least gross contamination or presence of racemic amino acids can be detected.

Much discussion exists among chemists as to the requirements for purity of DCM to be used in SPPS. Many chemists use commercial technical DCM directly from 55-gallon drums, while at the other extreme, some chemists insist on use of only highly purified spectro-quality DCM. Analytical reagent grade DCM appears to be repackaged technical DCM. Unless some contamination has occurred after the material has left the plant, technical DCM is of high quality. It is possible, however, to obtain DCM contaminated by water or HCl. Additional discussion of solvent quality is given in Chapter 2.

Commercially available TFA appears to be generally satisfactory for SPPS, especially if it is used with a scavenger. Both material obtained from Halocarbon in 5-gallon drums and analytical reagent grade TFA have been used. TFA is extremely hygroscopic, and can become wet if opened repeatedly. Furthermore, decomposition to trifluoroacetaldehyde can occur. These problems are not readily corrected by distillation, since TFA is readily converted to the anhydride by heat. Use of fresh material and care in handling are the best procedures. Impurities in TFA can be especially harmful in synthesis of peptides

containing tryptophan. Use of indole-N-formyl tryptophan has been recommended to overcome these problems, but removal of the formyl group requires treatment of the finished peptide with alkaline solution; this treatment can hydrolyze labile amide groups if such are present in the peptide, and should be avoided if possible. Use of a scavenger in the TFA reagent is highly recommended, and will serve to protect tryptophan, as well as other reactive groups in the peptide, from any harmful substances in the TFA. It now appears that the formyl group can be removed from tryptophan by thiolysis during HF cleavage if a low concentration of HF in dimethyl sulfide is used in the new procedure of Tam, Heath and Merrifield.[8a] See the section on HF cleavage for a more complete discussion.

Neutralization of peptide-resins by treatment with TEA solutions has been standard practice since the beginning of SPPS. Fresh reagent grade TEA is generally satisfactory; amine which has any color should not be used. TEA reacts with halogenated solvents such as DCM and CHL; carbenes are formed in this reaction. Although such carbenes are potentially harmful to peptides, no accounts of specific damage to peptides have appeared. In any event, TEA solutions in DCM or CHL should be freshly prepared.

Cleavage of peptide-resins by HF has become almost universal with the commercial availability of HF in cylinders and of a suitable vacuum line for carrying out cleavage reactions. While HF is an extremely dangerous substance, there is no need for any persons to be harmed if reasonable precautions are followed in carrying out cleavage reactions. These precautions are described in the experimental part of this book (Chapter 2) and should be followed in detail. HF reactions have been carried out in the authors' laboratories for nearly 20 years without any accidents.

B. THE POLYMER SUPPORT

A suitable insoluble support and a satisfactory means of attaching the first amino acid to it are of critical importance for successful SPPS. The solid support must be in particles of a physical size and shape that will permit ready manipulation and rapid filtration from liquids. It must be inert to all the reagents and solvents used during the synthesis of peptides, yet it must be modifiable in some way that will allow ready attachment of the first amino acid residue to it by a covalent bond. The polymer must swell extensively in the solvents used for synthesis, allowing all reagents to penetrate readily throughout the particles of the polymer. The polymer should not interact in any harmful way with any reagents or with the peptide chain in a way which will interfere with satisfactory assembly of the desired amino acid sequence into the peptide chain.

1. Cross-linked Polystyrene

The solid support chosen by Merrifield after a long search was composed of fine beads (20-50μ in diameter) of a synthetic resin prepared by copolymerization of styrene with a low percentage of divinylbenzene. The resin originally used by Merrifield contained 2% DVB, but later work showed that a resin prepared with 1% DVB had a greater degree of swelling in DCM and DMF, and the reactions of SPS appeared to go better in this latter resin. If a degree of cross-linking much lower than 1% is used, the resin is very soft and cannot be manipulated in the usual way. Birr[9] has used a resin made with 0.5% DVB, but that resin is extremely soft and cannot be filtered. He designed and used a centrifugal reactor with this soft resin, and has reported very good results with its use. The extreme case, of course, would be the use of a linear polymer of styrene, without cross-linking, but that material is soluble in the usual organic solvents and filtration can no longer be used for separation of the peptide-resin from soluble by-products. The soluble polymer system has been studied by Bayer,[10] but it will not be further discussed in this book.

The standard 1% cross-linked resin is a commercial product prepared as a starting material for synthesis of ion-exchange resins. Functional groups are introduced into this polymer by chloromethylation, usually with methylchloromethyl ether. Since methylchloromethyl ether has been reported to be carcinogenic, investigators should approach performance of the chloromethylation reaction with considerable

caution. Indeed, the ether is not generally available commercially at the present time. Chloromethylation can also be carried out with formaldehyde and HCl, but the better procedure is to purchase already chloromethylated resin. A suitable degree of chloromethylation for SPPS is 0.5 - 0.7 mmole chlorine/g of resin.

The chloromethylation reaction must be done with care. If the chloromethylation reaction is prolonged excessively or if the temperature is too high, chloromethyl groups alkylate other phenyl rings in the polymer and introduce additional cross-links, with elimination of chlorine. For this reason, a simple measurement of the amount of chlorine in the polymer is not always an indication that the resin will be satisfactory for SPPS. A rough measure of the degree of cross-linking of the polymer can be obtained by studying the degree the resin swells in DCM (see Chapter 2). If a resin with a suitably low degree of chlorine substitution does not swell adequately in DCM, the chloromethylation was probably carried out too vigorously, and the resin may not yield good results in synthesis.

2. Polyamide Resins

During the early years of SPPS some peptide sequences were encountered which proved to be essentially impossible to synthesize with good yield. A significant percentage of the growing peptide chains apparently terminated and would no longer couple with new activated Boc-amino acids. Sheppard[11] suggested that the cause of this difficulty was an incompatibility between the natures of the growing peptide chain and the polystyrene resin. While the resin is markedly hydrophobic, the peptide chain is fundamentally hydrophilic. Solvents commonly used for SPPS, such as DCM, swell the resin effectively but would not be expected to solvate peptide chains very well; Sheppard suggested that in such solvents the peptide chain might collapse upon itself and no longer be reactive. Conversely, solvents which would solvate the peptide chain effectively and open it up for continuation of synthesis would be expected to collapse the polystyrene matrix, preventing adequate access of reagents to the growing peptide chains. A solvent such as DMF, which solvates peptide chains and also swells polystyrene, might be expected to be a useful compromise to avoid this problem, but DMF does give rise to certain problems during SPS. In attempting to overcome these difficulties, Sheppard developed a cross-linked polyamide resin, whose fundamental nature was very similar to that of peptide chains. It was thus to be expected that a single solvent might effectively solvate both the peptide and the carrier matrix. Using the best of current SPPS technology, Sheppard and his associates showed that this polyamide resin made possible the synthesis of certain very difficult sequences. An outstanding example was the C-terminal region of acyl carrier protein, ACP (65-74). This peptide had been used by several investigators as an example of an "impossible" sequence for SPPS. In their work the Sheppard group used dimethylacetamide as solvent and a large excess of Boc-amino acid symmetric anhydride as the coupling agent, making the synthesis extremely expensive. In response to this challenge, Kent and Merrifield[12] studied the synthesis of ACP(65-74) extensively and showed that using similar excellent reaction conditions, the polystyrene and polyamide resins gave comparable good results. This study appears to be the only example of a careful comparison of polystyrene and polyamide resins, and on the basis of these results the added expense of the polyamide resin would not seem to be justified. Results may be quite different with other peptide sequences. The recent introduction by Sheppard of a polyamide resin supported on porous kieselguhr opens new possibilities for continuous-flow synthesis systems (see chapter 3).

C. THE PEPTIDE-RESIN LINK

1. The "Classical" SPPS System

For the synthesis of peptides having free carboxyl groups, the C-terminal amino acid is attached to the polymeric carrier as an ester (see Figure 1-4). The original Merrifield method for this esterification involved heating the TEA salt of a Boc-amino acid in ethanol with the chloromethyl resin. This reaction is quite slow, and the mixture is usually refluxed for one or two days. It is usually not possible to replace all of the

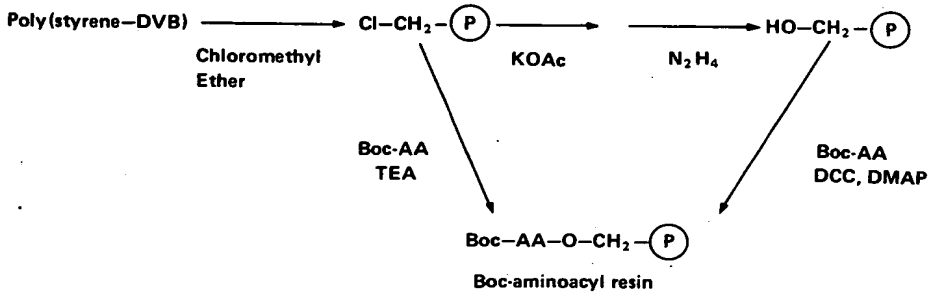


FIGURE 1-4.
Attachment of the First Protected Amino Acid to the Resin.

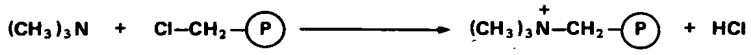


FIGURE 1-5.
Formation of Quaternary Ammonium Groups on the Resin.

chlorine in the polymer with Boc-amino acid. This esterification reaction proceeds faster in DMF than in ethanol, consistent with the known ability of DMF to promote nucleophilic reactions. An interesting variant is the use of KF as a catalyst for this esterification.

One undesirable side reaction in the classical esterification procedure is the formation of quaternary ammonium groups on the polymer (see Figure 1-5). These quaternary groups give the polymer characteristics of an anion-exchange resin, and may cause problems during the synthesis. If the chlorine on the resin is not completely replaced by Boc-amino acid, this quaternization can continue to occur at every neutralization step during the synthesis. While many peptides have been synthesized with completely satisfactory results when the first amino acid was attached in the classical way, most investigators prefer to use other procedures, especially if the peptide is large. The classical procedure continues to be used, however, because of its simplicity. If the Boc-amino acid is first converted to a tetraethylammonium salt or to a cesium salt, these reagents can be used to achieve essentially complete replacement of chlorine on the resin.

Several other kinds of problems arise from the use of chloromethyl resins. Amino acids which contain easily alkylatable functional groups cause difficulty in the esterification reaction. Such amino acids are histidine, cysteine and methionine. If ethanol is used as the solvent for the esterification reaction, some ester interchange might be expected to occur with aspartic and glutamic acids; these usually have their side-chain carboxyl groups blocked as benzyl esters.

All these problems can be avoided by using a hydroxymethyl resin for attachment of the first amino acid (see Figure 1-4). Although some additional work is involved in synthesis of this resin from chloromethyl resins, the work seems justified. The first Boc-amino acid can then be attached to the hydroxymethyl resin in a variety of ways, the most popular being by means of a DCC-mediated esterification reaction. Since ester formation is not very rapid with DCC, dimethylaminopyridine is usually used as catalyst to accelerate the reaction. One caution is that DMAP has been reported to cause partial racemization of amino acids when it is used in coupling reactions. For this reason, one should probably use only catalytic amounts of DMAP. Before an aminoacyl resin prepared in this way can be used for synthesis, all remaining hydroxyl groups on the resin must be blocked by acylation. Benzoylation has commonly been used for this purpose, but acetylation is probably satisfactory.

Whatever means is used for attaching the

first amino acid to the resin, the degree of substitution of this amino acid must be determined before peptide synthesis is begun. Amino acid analysis is the only fully satisfactory method for determining the degree of substitution. The aminoacyl-resin is first hydrolyzed with HCl and propionic acid. Aqueous constant boiling HCl is not satisfactory, because it does not wet and penetrate the pores of the resin. It is important when using the HCl-propionic acid procedure to evacuate the resin first in the propionic acid to eliminate trapped air from the beads and wet the resin with the propionic acid. Concentrated HCl is then added and the tube closed; the hydrolysis is carried out at 130°. Considerable pressure is generated during the heating, since the solution is supersaturated with HCl. (see p. 108).

2. Stable Peptide-Resin Links

As was mentioned above in the section on strategy, the ester linking the peptide to the resin in the classical SPPS system is slightly labile to the reagents normally used for removal of Boc groups at each step of the synthesis. When 25% TFA in DCM is used for deprotection, this loss may be about 1% per deprotection step. While this loss may not be of serious consequence in the synthesis of short peptides, it is not acceptable for synthesis of long peptides. If the Boc group is to be used as the α-protecting group during the synthesis of a long peptide, a more stable peptide-resin link is needed than that afforded by the classical system. This increased stability can be obtained by substitution of the resin with electron-withdrawing groups. Merrifield originally showed that nitration or bromination of the polymer gives peptide-resin links which are much more stable to acidolysis than in the case of the classical system. The stabilization obtained by nitration is too great for satisfactory SPPS; the peptide-resin link is not cleaved by acidolysis with HF or HBr. A brominated resin has been used with success in at least one synthesis, but another investigation[13] suggested that the brominated resin might not be satisfactory for general application. The brominated resin does not swell extensively in DCM and DMF, and reagents probably do not penetrate the beads very well.

The Pam resin introduced by Merrifield[14] increases the stability of the peptide-resin link by a factor of 100 over that of the classical system. This increased stability appears to make Pam resin the one of choice for synthesis of long peptides with Boc-amino acids. The increased stability of the peptide-resin link in Pam resin is due to the electron-withdrawing effect of the acetamido group in the para position of the phenyl ring to which the peptide is attached. Since synthesis of Pam resin involves considerable work (Figure 1-6), its use is probably not justifiable for synthesis of short peptides. The procedure recommended by Merrifield for synthesis of Pam resin is shown in Figure 1-6. The Boc-amino acid which will become the C-terminal residue of the peptide is esterifield to *p*-bromomethylphenylacetic acid phenacyl ester. This complex ester **(4)** is then treated with zinc and acetic acid for reductive cleavage of the phenyl ester, leaving the Boc-amino acid esterified to *p*-hydroxymethylphenylacetic acid **(5)**. This combined amino acid-linking group is then attached to an aminomethylpolystyrene by the use of DCC to give the starting material for peptide synthesis, the Boc-aminoacyl Pam resin **(6)**. Use of this procedure means that this reaction sequence must be completed for every amino acid which will be used as the C-terminal residue of a synthetic peptide. This involves a great deal of work. It does, however, have the advantage that no other reactive groups are left on the resin to cause problems with side reactions during the synthesis.

A great deal of time can be saved if the alternate Pam synthesis shown in Figure 1-7 is used. In that procedure, an aminomethyl resin is acylated with *p*-acetoxymethylphenylacetic acid **(7)** by the use of DCC. The acetyl group is removed by hydrazinolysis to yield a hydroxymethyl Pam resin **(9)**. The Pam resin can then be used as a starting material in synthesis and acylated with a variety of Boc-amino acids by the use of DCC and DMAP, in a manner completely analogous to that used with the hydroxymethyl classical Merrifield resin. Regardless of which procedure is used in synthesis of Boc-aminoacyl Pam resins, unreacted amino or hydroxy groups

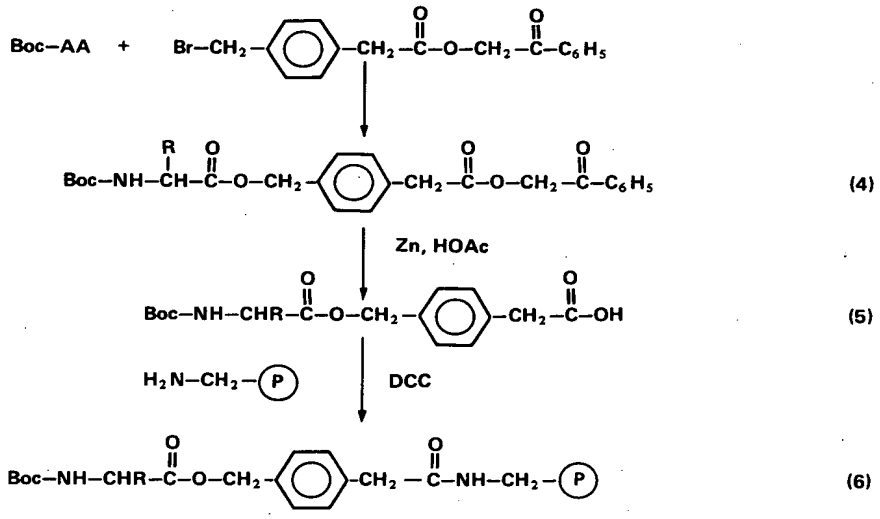


FIGURE 1-6.
Synthesis of Boc-aminoacyl Pam Resin. The Merrifield Method.

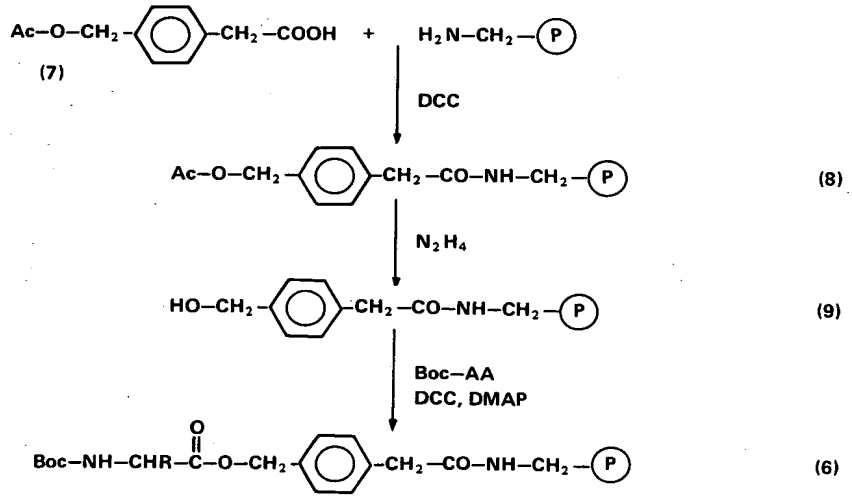


FIGURE 1-7.
Synthesis of Boc-aminoacyl Pam Resin. Alternate Method.

on the resin must be blocked by acylation before peptide synthesis is begun. Use of Pam resins has recently permitted successful synthesis of several quite large peptides. Other aspects of the use of Pam resins are discussed in the section below on side reactions (K).

3. Labile Peptide-Resin Links

Generally, as peptides become larger and more complex they become less stable toward harsh reagents such as HF and TFA. For this reason several chemists have investigated the question of more labile peptide-resin links for

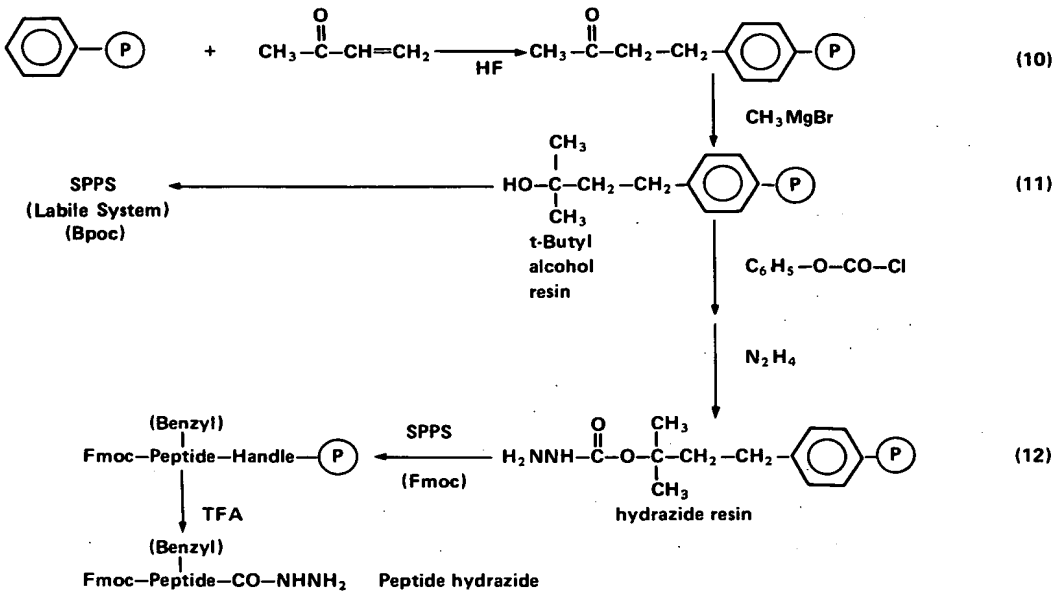


FIGURE 1-8.
The Wang Labile Resin Systems.

use in SPPS. Such labile links should allow final cleavage of the peptide from the resin by a milder reagent, such as 25% TFA in DCM. One such resin is the *p*-alkoxybenzyl alcohol resin of Wang.[15] In this resin (3), the peptide-resin link is labilized by the electron-donating ability of the ether oxygen in the para position of the ring. Peptides attached to this resin may be cleaved by TFA-DCM. For synthesis with this resin, one must use α-blocking groups which are extremely labile, such as the Bpoc group (see Section D below). If side-chain functional groups are blocked by *t*-butyl-derived blocking groups, side chain deblocking can be accomplished at the same time the peptide is cleaved from the resin with TFA. While this synthetic strategy does indeed eliminate exposure of the final peptide to extremely strong acidic reagents such as HF, HBr, or TFMSA, the peptide is exposed to a large concentration of *t*-butyl carbonium ions during the cleavage reaction. In some cases, this may be even more harmful than the conditions of the standard cleavage with HF. For example, tryptophan is very readily alkylated under these conditions to yield peptide products containing *t*-butyltryptophan groups (see Section K). Use of this system also incurs the added complications of working with the extremely labile Bpoc-amino acids, which must be stored as salts. The entire range of Bpoc-amino acids needed may not be commercially available, and if available, they are extremely expensive.

4. Synthesis of Blocked Peptides

The use of segment SPPS was discussed above in the section on strategy as a means of reducing heterogeneity in the final product during synthesis of long peptides. For this purpose, the short blocked peptides which will be assembled on the solid phase polymer must first be synthesized. This has commonly been done in solution,[7] but by proper design of a solid phase system; such blocked peptides can be prepared by SPS. Three such systems are indicated in Table 1-I. If the ether resin of Wang[15] is used with Fmoc-amino acids in which the side-chain functions are blocked by benzyl-related groups, blocked peptides can be cleaved from the resin with TFA and after purification, used for further synthesis. Figure 1-8 shows a series of reactions

used to synthesize a *t*-butyl resin which can be used in a similar fashion.[16] As shown in Figure 1-8, this *t*-butyl resin can be further modified to a hydrazide resin, which can be used in a similar system of SPPS to yield blocked peptide hydrazides after cleavage from the resin with TFA. These hydrazides would then be ready for coupling by the azide procedure.

In all such work where blocked peptides will be coupled to the resin in SPPS, the chemist must remain aware of the hazards of racemization of the C-terminal residue of such peptides when they are activated for coupling to the peptide-resin. A particularly advantageous feature of stepwise coupling in SPPS is that single amino acids protected by urethane α-protecting groups do not racemize significantly when activated by the usual reagents. These urethane blocking groups apparently prevent cyclization of the activated derivative to an azlactone, with concomitant racemization before the azlactone couples in the synthetic step. When the carboxyl group of a blocked peptide is activated, however, racemization can occur unless the C-terminal residue is glycine or proline. If segment coupling is to be used in SPPS and the peptide being synthesized has many glycine and proline residues, the synthesis can be broken at these residues without danger of racemization. Such was the case in the synthesis of a collagen analog peptide by Sakakibara.[17] The azide coupling reaction has long had the reputation of providing racemization-free synthesis. This dogma, however, has been challenged in recent years with the demonstration of some racemization in certain azide couplings. Apparently, it is not always safe. The hydrazide resin was designed to take advantage of the good features of azide coupling.

5. Resins for Synthesis of Peptide Amides

If a peptide is synthesized on an aminomethyl resin (such as that used as starting material for synthesis of Pam resin, see Figure 1-7) the resulting peptide-resin link will be found to be completely stable to acidolysis. On the other hand, if the peptide-resin link is activated by addition of another phenyl ring, the resulting peptide-resin will be found to be labile to HF, and peptide amides will result from this cleavage (see Figure 1-9). The first such resin was synthesized by Pietta and Marshall,[18] who acylated cross-linked polystyrene with benzoyl chloride to give a ketone resin **(13)** which could be reductively aminated to yield a benzhydrylamine (BHA) resin. This resin has been used successfully for synthesis of many peptide amides. It was found, however, that some peptides with C-terminal phenylalanine or leucine could not be removed from the resin by HF at 0°. Investigation revealed that the stability of the peptide-resin link depends markedly upon the nature of the C-terminal residue. Matsueda and Stewart[19] studied a series of acetyl-amino acid BHA resins and found the order of increasing stability of a series of amino acids to be His, Gly, Thr, Glu, Pro, Met, Val, Leu, Phe (most stable). They introduced the MBHA resin **(14)**(X = — CH_3), which gave considerably more labile peptide-resin links, due to the electron donating property of the methyl group on the introduced ring. Synthesis of this amine resin involved acylation of polystyrene with toluyl chloride followed by Leuckart reduction. This MBHA resin has been used with satisfactory results for a wide variety of syntheses of peptide amides. Other investigators[20] suggested a methoxy-BHA resin for synthesis of peptide amides, but our investigations suggested that the peptide-resin link in this resin was too labile for synthesis of most peptides when α-Boc protection is used. The methoxy-BHA resin should be useful in conjunction with more labile α-protecting groups.

D. PROTECTION OF α-AMINO GROUPS

1. Acid Labile Urethanes

The most popular α-protecting group during the last two decades has been the Boc group. As mentioned above, the very satisfactory lability-stability characteristics of this group, in connection with the ready commercial availability of derivatives which are, for the most part, satisfactory for general peptide synthesis, has contributed to this popularity. The Boc group itself is sufficiently stable that the amino acid

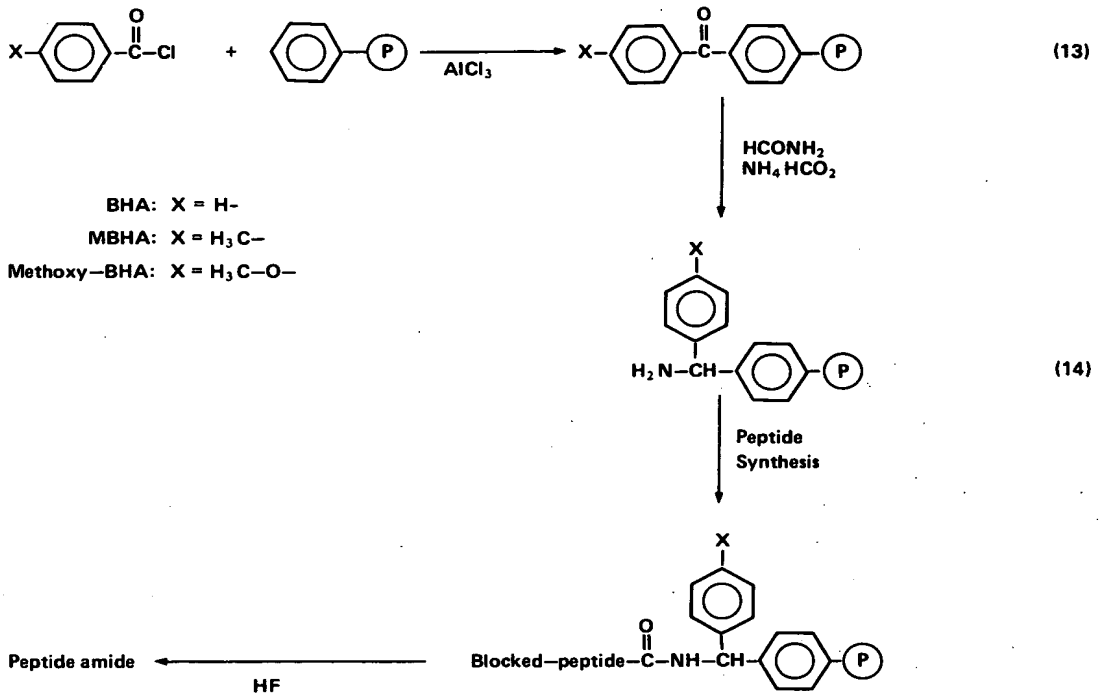
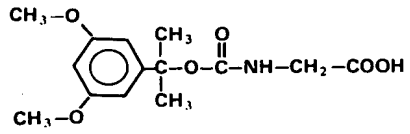


FIGURE 1-9.
Synthesis of Peptide Amides *via* Amine Resins.

derivatives can be stored at room temperature, although most chemists prefer to keep them in the refrigerator. The one general exception is Boc-His(Tos), which is not adequately stable at room temperature, and must be purchased as the DCHA salt and converted to the free acid before use in synthesis. For those investigators who have HF apparatus available, the complete set of needed side-chain derivatives is also available. Some desirable additional changes in the area of side-chain blocking groups are discussed below. This general acceptance of the Boc group, however, does not mean that it is ideal. As discussed above, the differential stability of the Boc α-protecting group and the peptide-resin link of the classical Merrifield resin is not adequate for synthesis of long peptides. While the use of Bpoc would, at first, appear to be ideal to improve this situation, this is not so, practically. The principal disadvantage is that the Bpoc group is so labile that the derivatives are not stable as the free acid, and must be stored as a salt. This requires conversion to the acid immediately before use in synthesis. While this is no significant problem for investigators doing manual synthesis, it is a serious disadvantage when automatic synthesizers are used, since the free acid is not stable in solution during the several hours which may elapse between preparation of the solution and the actual use in coupling when automatic synthesizers are run overnight or over weekends. The difference in reactivity to acidolysis by TFA between Bpoc and Boc is approximately 120,000. While the Bpoc is too labile for convenient general use, other derivatives of intermediate reactivity have been designed and are stable enough that they can be stored as the free acid but are still labile enough that more dilute solutions of TFA can be used for deprotection; these dilute TFA solutions should not harm the peptide-resin link in the classical system.



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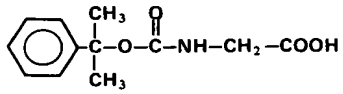

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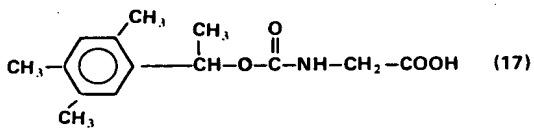

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Three such groups are the Ddz group **(15)** of Birr,[21] the Poc group **(16)** studied by Ragnarsson[22] and the Tmz group **(17)** proposed by Matsueda and Stewart.[23] The Tmz group has, in addition to an ideal lability to acidolysis, the feature of optical activity. If one synthesizes Tmz-amino acids with an optically pure Tmz reagent, then simple crystallization of the Tmz-amino acid to chemical purity would insure optical purity of the derivative. This feature should be a marked benefit for synthesis of optically pure peptides. The ready availability of starting materials and ease of synthesis of the derivatives are additional benefits for this derivative. In contrast, the Ddz derivatives are very expensive, and the Poc derivatives are not commercially available. Commercial availability at a reasonable price is a critical factor in general adoption of a set of amino acid derivatives for peptide synthesis.

Practical useful deprotection of Bpoc amino acids can be obtained by 30-minute treatment with 0.2% TFA in DCM, while Ddz, Poc or Tmz amino acids require two or three percent TFA for 30 minutes. Either of these deprotection regimes should cause no harm to the peptide-resin ester link in the classical Merrifield system.

2. Base Labile Groups

The principal base-labile α-protecting group to have found a place in SPPS is the Fmoc group.[11,24] This derivative **(18)** is usually removed from amino acids and peptides by treating them with a solution of a secondary amine in DMF (see Figure 1-10). Use of Fmoc amino acids provides a desirable orthogonal system for SPPS, and it has been used for the synthesis of several peptides. One caution in the use of Fmoc amino acids is that the classical Merrifield peptide-resin link will probably not be stable to the concentrated solutions of amines necessary to remove the Fmoc group. This loss may produce undesirable short N-terminal sequences of the peptide being synthesized due to attachment of amino acids to the exposed hydroxyl groups, thus starting new peptide chains. This problem is usually overcome by the use of an acid-labile (and consequently base-stable) resin, such as the ether resin (see Section B.3 above). If a given coupling reaction is slow when Fmoc amino acids are being used, there is always a risk of removal of the Fmoc group by N-terminal amino groups in the peptide-resin and subsequent coupling of a second Fmoc-amino acid onto the peptide-resin. This side reaction will lead to formation of undesirable insertion sequences. As pointed out above, another difficulty which has slowed general acceptance of the Fmoc-amino acids for SPPS is the high cost of the derivatives and unavailability of the complete set of most desirable derivatives for SPPS.

Since the acid-labile ether resin should be used for Fmoc SPPS, final cleavage of the peptide can be carried out by treatment with 25% TFA in DCM. Consistent with this system is the use of *t*-butyl derived side chain blocking groups for trifunctional amino acids.

3. Other α-Protecting Groups

Among the other types of α-protecting groups available, the Nps group **(19)** has been used to some extent in SPPS. This group can be removed from amino acids by either very dilute anhydrous acid or by nucleophiles (see Figure 1-11). Treatment of an Nps peptide or Nps amino acid with HCl causes formation of Nps

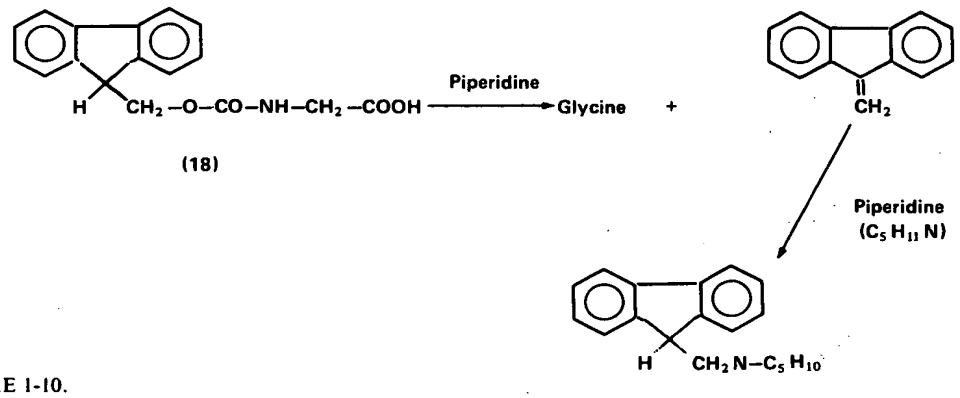


FIGURE 1-10.
Cleavage of Fmoc-glycine by Piperidine.

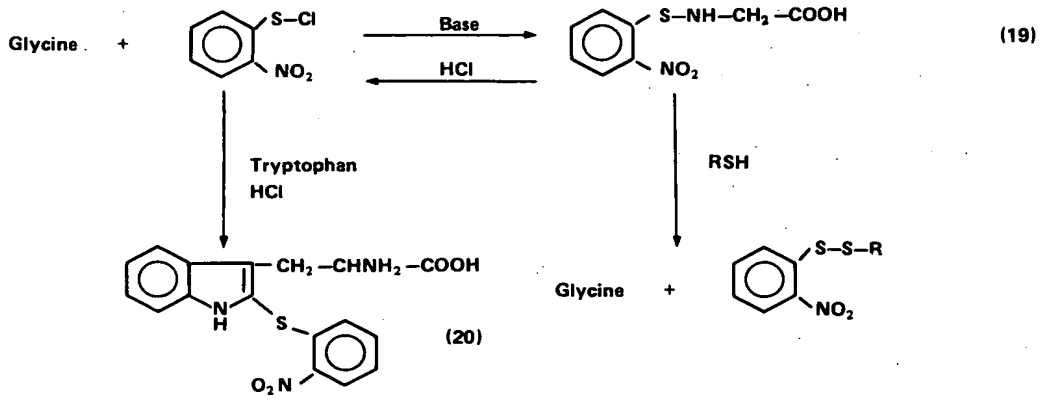
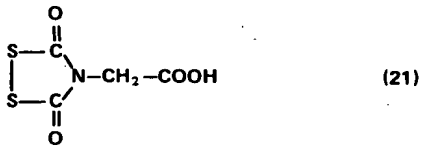


FIGURE 1-11.
The Nitrophenylsulfenyl Protecting Group.

chloride as by-product; this is essentially a reversal of the reaction used for synthesis of Nps derivatives. This scheme of deprotection can be used satisfactorily unless the peptide contains tryptophan. In the latter case, the Nps chloride formed in the deprotection step reacts with tryptophan irreversibly to form an indole-substituted thioether **(20)**. As is suggested by the reaction in Figure 1-11, reactions of Nps derivatives are equilibria and do not proceed to completion unless forced. In the case of HCl cleavage, the reaction is forced to completion because the free amino group formed is tied up immediately as the HCl salt. If one wishes to use Nps derivatives for synthesis of tryptophan-containing peptides, deprotection by treatment with a nucleophile can be used. In this case, the equilibrium nature of the deprotection reaction may be a problem. One report[25] described synthesis of a 30-residue peptide by SPPS using Nps amino acids and using methyl-3-nitro-4-mercaptobenzoate as deprotection reagent. Unfortunately, details of the procedure were not given.



Another interesting α-protecting group removable by nucleophilic attack is the dithiasuccinyl (Dts) group proposed by Barany and Mer-

rifield (21). Although preliminary work suggests that this may be a very useful protecting group for SPPS, little actual synthesis has been done as yet. The reader is referred to the Barany and Merrifield review[1] for a further discussion.

E. BLOCKING OF SIDE-CHAIN FUNCTIONAL GROUPS

1. The Standard System for Use with Boc Amino Acids

The system of benzyl-derived side-chain blocking groups originally used by Merrifield has continued to be most widely used in conjunction with Boc-amino acids, with some modifications. The exact derivatives most commonly used at the present time are discussed under the individual amino acids, below. These blocking groups can be removed from all amino acid residues by treatment with HF at the end of the synthesis. All of these are also removable by HBr or TFMSA except the Arg(Tos) group. Additional work is still needed to provide a somewhat more labile blocking group for arginine side chains. The tosyl group is also commonly used to block the imidazole ring of histidine, but this group also falls short of ideal. It is too labile, and a more stable blocking group needs to be developed.

An additional serious problem arises in certain peptide sequences from cyclization of aspartyl residues to succinimides which then open in the wrong direction to yield β-aspartyl peptides. This problem is also discussed below.

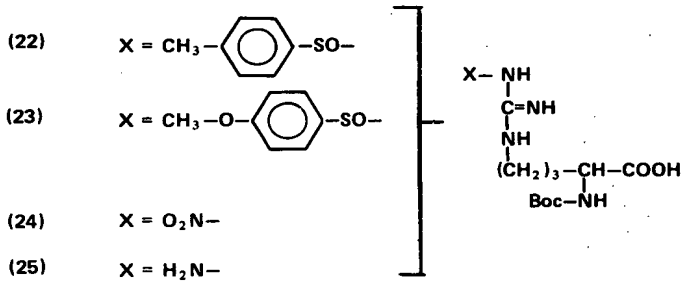
2. More Labile Systems

When very labile α-protecting groups are used, such as Bpoc, Poc, Tmz, or Nps with acidolytic removal, or Fmoc with base removal, the side-chain functions of trifunctional amino acids may be blocked by more labile groups. The most commonly used groups in this case are those based upon *t*-butyl blocking groups. If the acid-labile ether resin is used for the synthesis, cleavage and side-chain deblocking can be accomplished in one step at the end of the synthesis by use of 25% TFA-DCM. While this system avoids exposure of the finished peptide to strong acids such as HF or HBr, this system is not without its hazards. As mentioned above, tryptophan residues are subject to *t*-butylation during this cleavage, and extremely effective scavengers must be used to avoid this problem. The indole ring of tryptophan can also be blocked with the N-formyl group in this system, but the formyl group must be removed subsequently by treatment of the peptide with base, a procedure which may not always be without damage to the peptide. However, it now appears that the "low HF" procedure[8a] will successfully remove the formyl group (see section J. 1, below).

3. Blocking Groups for Individual Amino Acids

a. Arginine. The guanidine group in the side chain of arginine is extremely basic (pK = 12.5), but under certain conditions it can be acylated. In solution peptide synthesis the guanidine is often protected merely by being present as a hydrohalide salt. In SPPS, on the other hand, where a large excess of acylating group is always present, the guanidine group of arginine has traditionally been blocked for synthesis. The arginine derivative most commonly used at the present time for classical SPPS, when HF will be used for cleavage of the peptide-resin, is Boc-Arg (Tos)(22). The tosyl group on the guanidine is extremely stable, and can be removed practically only by HF. It is not removed by HBr or TFMSA. For those chemists with HF apparatus, use of this derivative has solved most of the problems associated with incorporation of arginine into peptides. If HF equipment is not available, the only practical method for removal of the tosyl group is reductive cleavage by sodium in liquid ammonia. While this latter reagent has been used with success for synthesis of many peptides, particularly those containing cysteine (blocked with S-benzyl groups), it can be harmful to some peptides, particularly those containing proline, which may be reductively cleaved. Successful use of Na-NH_3 requires considerable skill.

A somewhat more labile derivative of arginine is needed for SPPS. Yajima[26] has used methoxybenzenesulfonyl arginine (23) in solution synthesis. This blocking group has been removed satisfactorily by TFMSA, and could probably also be cleaved by HBr.



The guanidine group can also be protected by nitration, and Boc-nitroarginine was the derivative most commonly used in SPPS before advent of HF for cleavage. The nitro group **(24)** is not removed by HBr or TFMSA cleavage reagents, and must be removed by hydrogenolysis. Hydrogenolysis of nitroarginine-containing peptides can be frustratingly slow, and the intermediate aminoarginine **(25)** may persist in the final peptide. Aminoarginine is less basic than arginine. Boron tris-(trifluoroacetate) has been reported[27] to cleave the nitro group effectively, but this reagent may not be completely satisfactory as a solid phase cleavage reagent. Other cleavage reagents such as TFMSA and pyridine-HF appear to be less satisfactory.

One potential problem in the use of nitroarginine is its facile cleavage by base to yield ornithine (see Figure 1-12). Nitroarginine can thus not be used if the peptide will be cleaved from the resin by ammonolysis or amine-catalyzed transesterification. In certain cases, cleavage to ornithine has been reported to take place during the course of standard SPPS procedures. This latter problem is probably sequence dependent, but caution should be used when nitroarginine is incorporated into peptides.

Acid hydrolysis of peptide-resins containing nitroarginine yields a mixture of products. The derivative is converted partially to arginine, but some ornithine is also produced, as well as unhydrolyzed nitroarginine. These three prod-

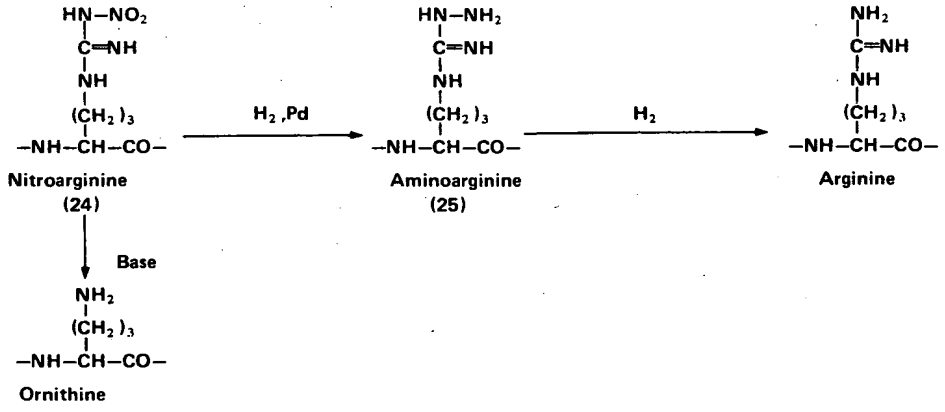
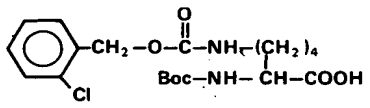


FIGURE 1-12.
Reactions of Nitroarginine.

ucts must be summed to obtain an accurate measure of the amount of arginine in the peptide.

The situation is less satisfactory when more labile systems of SPPS are to be used. The guanidine group of arginine can also be blocked as the di-Boc derivative, but some other problems may arise in this case. Further investigation of such derivatives is still needed.

b. Lysine. Benzyloxycarbonyl or substituted benzyloxycarbonyl groups have been consistently used for protection of the ϵ-amino group of lysine in classical SPPS. It was learned relatively early that the Z group is not adequately stable for synthesis of long lysine-containing peptides by SPPS, and negatively substituted Z groups were introduced to improve the stability. At the present time the 2-chlorobenzyloxycarbonyl **(26)** group is most commonly used, and appears to be quite satisfactory.



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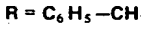
When a more labile system of SPPS is desired, α-Bpoc-Lys(Boc) can be used, with TFA as the final cleavage reagent. If one wishes to synthesize lysine-containing peptides for further reaction, such as synthesis of an antigenic peptide to be conjugated to a protein carrier, it is desirable to have the blocking group on the lysine side-chain remain intact during cleavage and purification of the peptide. The trifluoroacetyl group is satisfactory for this purpose, as it is not cleaved by HF. After the peptide has been conjugated to the carrier by means of a reagent such as EDC, which reacts with amino groups, the Tfa-Lys groups can be deblocked by treating the peptide-protein conjugate with aqueous piperidine.

c. Histidine. Several blocking groups have been used for histidine in SPPS, but even today all of the available groups leave something to be desired. At the present time Boc-His(Tos) **(28)** is the most commonly used derivative of histidine for SPPS. It usually gives satisfactory results if certain precautions are observed. TFA must be used for removal of Boc groups during synthesis, as the tosyl group is removed rapidly by HCl-dioxane or other mineral acids. This group is also marginally stable to base, and usually cannot be attached to chloromethyl resins in the initial stage of SPPS; hydroxymethyl resin should be used for this purpose. Furthermore, HOBt should not be used in coupling reactions, as it is acidic enough to remove the tosyl group. Certain sequences have been found in which tosyl histidine will not couple satisfactorily. If the common coupling reactions such as DCC and symmetrical anhydrides do not give complete coupling, the synthesis should be attempted using Dnp-histidine, as it has been found to give success in some of these difficult cases.

The use of Boc-His(Dnp) **(29)** requires an additional deblocking step in most cases, especially if acidolysis is used for final cleavage of the peptide from the resin; this group is stable to HF and HBr. Perhaps the most satisfactory procedure for removal of the Dnp group is treatment



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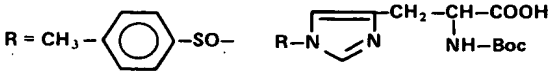

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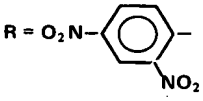


(29)

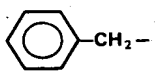
of the peptide-resin with thiophenol in DMF before cleavage of the peptide from the resin. This procedure leaves much yellow material adsorbed to the resin and contaminating the crude product, but since it is very hydrophobic, it can usually be separated from the peptide without difficulty. The Dnp group can also be removed from the peptide after cleavage by treating an aqueous solution of the peptide with 2-mercaptoethanol. The Dnp group is also labile to base, and is removed when a peptide is cleaved from the resin by ammonolysis. Boc-His(Tos) can be readily attached to hydroxymethyl resin without any difficulty. Some SPPS of histidine-containing peptides has been done without protection of the imidazole groups; this was true of the dramatic ribonuclease A synthesis of Gutte and Merrifield.[28] However, this procedure cannot be generally recommended, as other attempts to use unblocked histidine have been unsuccessful. An unblocked imidazole is readily acylated in peptide coupling reactions, and this will lead to waste of reagent in synthesis. Some coupling reactions may fail in the presence of unblocked imidazole groups.

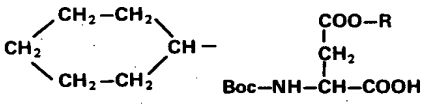
Initial syntheses of histidine-containing peptides by SPPS used imidazole-benzyl histidine **(27)**. The benzyl group does not neutralize the basic character (pK = 6.5) of the imidazole, but protects it by steric hindrance. This group is also stable to HF and HBr, and must be removed by subsequent hydrogenolysis or treatment with sodium in liquid ammonia, both of which are not universally successful. As pointed out above, Na-NH_3 can cause serious side reactions in the peptide, and catalytic hydrogenolysis for removal of this group is always very slow and can also cause concomitant saturation of aromatic rings in the peptide.

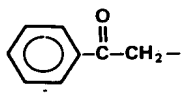
Another serious problem with the use of either unblocked histidine or imidazole-benzyl histidine is racemization. Since the basic character of the imidazole is not neutralized in either of these derivatives, it can lead to partial racemization of these derivatives when they are activated for coupling by DCC. Racemization is completely unacceptable in peptide synthesis and for this reason unblocked histidine and benzyl histidine must not be used.

It is clear from this discussion that all of the available histidine derivatives have some problems. Perhaps imidazole-benzenesulfonyl histidine should be tried; it should be more stable than the currently used tosyl derivative.

The most commonly used derivative, Boc-His(Tos), lacks fully adequate stability at room temperature; the tosyl group is slowly lost. While this difficulty can be overcome in the laboratory by storage of the derivative at refrigerator or freezer temperatures, shipping from commercial suppliers is a different matter, and degradation frequently occurs. For this reason, one should always purchase the derivative as a salt; the DCHA salt is commonly supplied. This salt is quite stable to shipping conditions, and can then be converted to the free amino acid derivative in the laboratory.

(30) R = 

(31) R = 

(32) R = 

d. Aspartic acid. The β-benzyl ester of aspartic acid has been used almost exclusively in SPPS in conjunction with Boc α-protection **(30)**. This derivative appears to be adequately stable for satisfactory synthesis of long peptides by the classical SPPS procedures. Yamashiro[29] suggested that the benzyl ester was not adequately stable, and suggested the use of the 4-bromobenzyl ester of aspartic acid to increase the margin of safety in selective acidolysis.

If the labile SPPS system based on α-Bpoc protection or the orthogonal system with Fmoc amino acids is to be used, the β-carboxyl of aspartic acid can be blocked as the *t*-butyl ester. This branched alkyl ester has the additional advantage that peptide amides may be synthesized using ammonolysis for peptide-resin cleavage without converting aspartic acid residues to

asparagine residues. This is not the case with the benzyl ester, which is converted to the amide.

When aspartic acid is followed in the peptide sequence by a glycine, alanine or serine residue, ring closure to succinimide and subsequent opening to yield the undesirable β-aspartyl peptide can occur. This side reaction is discussed in greater detail in section K, below. The cyclohexyl ester **(31)** and the phenacyl ester **(32)** have been proposed to help overcome this problem. The cyclohexyl ester is not subject to ready aminolysis,[30] in a manner analogous to the *t*-butyl ester. The phenacyl ester, on the other hand, can be removed prior to HF cleavage by treatment of the peptide-resin with sodium thiophenoxide in DMF.[31]

Most recently it appears that use of the "low HF" cleavage procedure of Tam, *et al.*[8a] can completely prevent formation of succinimide peptides. In this procedure HF and dimethyl sulfide are present in equimolar amounts (1:3 by volume), and the cleavage reaction proceeds by a different mechanism (see section J.1, below).

e. Glutamic acid. Glutamic acid residues have been blocked routinely as benzyl esters for classical SPPS in conjunction with Boc α-protection, and with *t*-butyl esters when the labile or orthogonal system will be used. Although ring closure to glutarimide peptides has not been a serious problem in SPPS, acylation of anisole during HF deprotection can occur to a significant extent. This side reaction is discussed below in section K.

f. Asparagine. When Boc-asparagine is activated with DCC for coupling in SPPS, dehydration of the side-chain amide group to the corresponding nitrile **(33)** can occur (see Figure 1-13). This problem has classically been avoided by use of an active ester, such as the *p*-nitrophenyl ester **(34)** in SPPS. Although DCC is used in synthesis of the active ester, any nitrile formed during this reaction can be removed by purification of the active ester before it is used in the coupling reaction. In general, active esters are much slower to react in coupling reactions than are DCC-activated intermediates. In SPPS, a larger excess of the derivative has been allowed to react for an extended period of time to overcome this problem. Even with these modifications, complete coupling of asparagine residues is not always obtained. An alternate approach has been the use of hydroxybenzotriazole active esters generated *in situ* by addition of HOBt to the DCC-mediated coupling reaction. In this situation, it is assumed that conversion of the DCC-activated intermediate (see Figure 1-19) to the HOBt active ester (which is a fast reaction) will occur before any significant dehydration to the nitrile (a slow reaction) can take place. Unfortunately, it is difficult to ascertain exactly how much

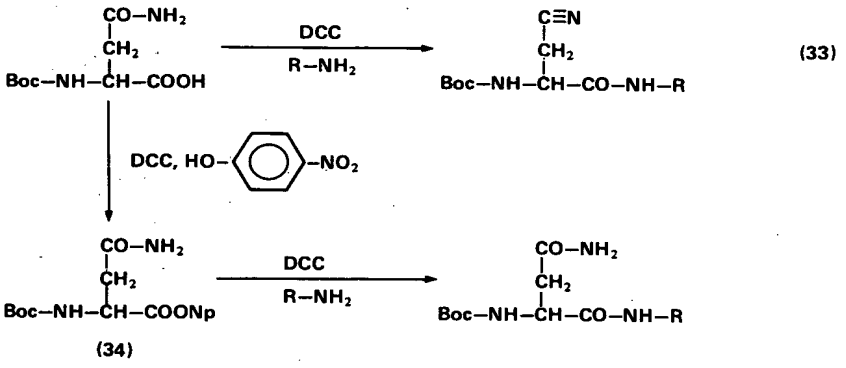
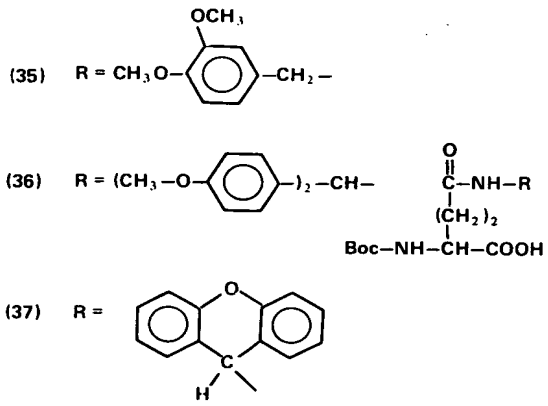


FIGURE 1-13.
Dehydration and Coupling of Asparagine (Glutamine is Analogous).

nitrile might have been incorporated into a peptide by this procedure. Total enzymatic hydrolysis of the peptide is probably the only reliable way to make an exact determination. As was mentioned above, the HOBt procedure should also be avoided if the peptide contains tosyl histidine groups, since the tosyl group is removed by HOBt.

Although symmetrical anhydrides often give superior results as coupling agents in SPPS, they cannot be recommended for use with asparagine (and evidently glutamine, as well). It has been shown[31a] that the Boc-Asn symmetrical anhydride will lead to nitrile formation during coupling



If the side-chain amide of asparagine (or glutamine) is alkylated with a removable blocking group, then dehydration to the nitrile is not anticipated. Unfortunately, no completely ideal group for this purpose is readily available. The N-dimethoxybenzyl derivative **(35)** appears to have a good reactivity, but the derivative is not commercially available and is difficult to synthesize. The 4,4′-dimethoxybenzhydryl group **(36)** seems to have a good reactivity, and should be explored further, since dimethoxybenzhydrol is commercially available. The 9-xanthenyl derivative **(37)** has been used to a considerable extent, and is commerically available. It is also extremely easy to synthesize from xanthydrol and Boc-asparagine. Unfortunately, it is too labile for ideal use in classical SPPS, since it is removed to a large degree by a single TFA deprotection step. However, it does provide the necessary protection against dehydration during the coupling step. An additional problem with the use of Boc-Asn(Xan) is the very low solubility of the N-acyl urea produced as a by-product in the DCC-mediated coupling reaction. This acyl urea precipitates in a very fine form and causes serious plugging of fritted disks in reaction vessels. While this is not difficult for manual SPPS, it is a serious problem in automatic synthesis; the operator should be present to add additional DMF to dissolve the acyl urea and avoid problems with the synthesizer due to poor filtration. All these derivatives are very bulky, and may cause problems in obtaining complete coupling due to steric hindrance.

The xanthenyl derivative of asparagine would appear to be satisfactory for use in labile or orthogonal systems, since its great acid lability is not a problem in these cases.

g. Glutamine. All the comments in the preceding section on asparagine apply equally to use of glutamine in SPPS. The same derivatives and procedures applied to elimination of asparagine problems have also been applied to use of glutamine, with similar results. One difference is that the N-acyl urea formed from Boc-Gln(Xan) is not so insoluble as that formed from Boc-Asn(Xan).

An additional serious problem occurs with glutamine peptides after removal of the Boc protecting group and before coupling of the subsequent residue (see Figure 1-14). N-terminal glutamine peptides **(38)** cyclize spontaneously to the pyroglutamyl peptide **(39)**. Use of N-alkyl amides of glutamine will avoid this problem. Other aspects of this problem are discussed in section K, below. This problem is also serious in labile SPPS work, since the dilute TFA used to remove Bpoc or Poc groups favors cyclization to the pyroglutamyl peptide.

h. Pyroglutamic acid. Many naturally occurring peptides (and proteins) appear in their native form with N-terminal pyroglutamyl residues. While several different approaches to synthesis of such peptides have been used, the simplest and

(38) (39)

FIGURE 1-14.
Formation of Pyroglutamyl Peptides.

most direct is the best: direct coupling of pyroglutamic acid by DCC. DMF must be used in this coupling reaction due to the low solubility of pyroglutamic acid. Some investigators have used benzyloxycarbonyl-pyroglutamic acid because of its increased solubility. While this can be done, it is not necessary. One must not use Z-<Glu if the peptide will be cleaved from the resin by ammonolysis; this will open the pyrrolidone ring and yield an N-terminal glutamine peptide.

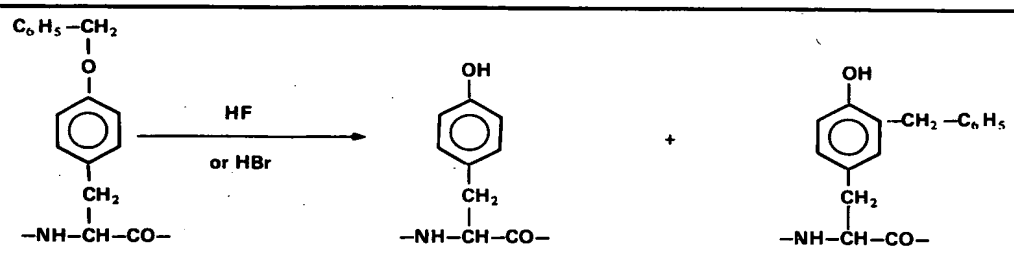
i. Serine. Serine has routinely been protected as the benzyl ether in classical SPPS. The benzyl ether is of adequate stability for all but perhaps the very longest peptides. For greater stability, halogen-substituted benzyl ethers can be used, but these are not commercially available at the present time. All of these benzyl ethers are cleaved satisfactorily by HF or HBr.

For labile SPPS work, the *t*-butyl ether can be used. The hydroxyl group of serine (and threonine) must be blocked in SPPS to avoid branching of the peptide chain on the hydroxyl group.

j. Threonine. The problems involved in the use of threonine in SPPS are quite analogous to those considered above for serine, and the blocking groups which have been used are the same. Yamashiro[29] has recommended *p*-chlorobenzyl threonine for increased stability in the synthesis of long peptides or proteins. For the labile systems of SPPS using threonine, the *t*-butyl ether has been used with success.

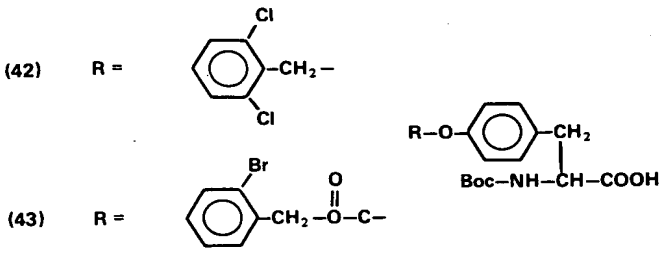
k. Hydroxyproline. This amino acid is of interest chiefly in collagen-related peptides and as a replacement for proline in peptide hormone analogs. The O-benzyl ether of 4-hydroxyproline (the naturally occurring isomer) is commercially available. Surprisingly, hydroxyproline has been used successfully in SPPS without protection of the hydroxyl group;[32] DCC was used as coupling agent in that work.

l. Tyrosine. The hydroxyl group of tyrosine must be blocked for satisfactory incorporation of this amino acid in SPPS. The O-benzyl ether **(40)** was originally used for this purpose, although it



(40) (41)

FIGURE 1-15.
Alkylation of the Tyrosine Ring by Benzyl Groups.



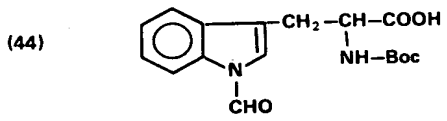
was early known that there were significant problems with the use of this derivative. It was later shown that the benzyl group migrated very readily to the 3-position of the tyrosine ring to yield a stable undesired product. **(41)** The benzyl ether is also cleaved to a significant degree by TFA during deprotection of Boc groups. The 2,6-dichlorobenzyl ether **(42)** is much more stable, and is widely used at the present time. Under some circumstances, it tends to yield some of the undesirable 3-alkylated derivative.

The alkylation problem can be totally avoided if a urethane group of proper stability is used to block the hydroxyl group of tyrosine. The 2-bromobenzyloxycarbonyl derivative **(43)** is now available for this purpose.

For labile systems of SPPS, the O-*t*-butyl ether has been used extensively, and appears to be quite satisfactory. It is readily removed by treatment with TFA or dilute anhydrous HCl.

Additional discussion of tyrosine side-chain reactions is included in section K, below.

m. Tryptophan. Although tryptophan is of great interest for synthesis of peptide hormones and their analogs, two types of problems must be overcome before it can be used successfully in SPPS. The first of these is due to the rapid oxidation of tryptophan in acidic media; the second is due to facile alkylation of the indole ring by carbonium ion reagents during cleavage. The N-formyl **(44)** derivative has been proposed and used extensively to overcome both these problems. The formyl group is stable to acidic cleavage reagents, and must be removed subsequently by treatmet with aqueous base. In recent work,[33] Merrifield found that treatment of an N-formyl tryptophan peptide with 0.03 M hydroxylamine, pH 9.0, for two hours appeared to be the best procedure available for removal of the formyl group. Others have raised the pH of an aqueous solution to 11 and rapidly returned it to neutrality. In any such procedures where the peptide is exposed to aqueous base, one might anticipate harm to some asparagine and glutamine amides; this is most likely if they occur in basic peptides adjacent to arginine or lysine residues. A better alternative for removal of the formyl group is use of the new "low HF" procedure[8a] (see section J, below).



Removal of the formyl group can be monitored by following loss of the specific absorbance at 300 nm.

It was already known some years ago that tryptophan could be alkylated by *t*-butyl carbonium ions formed when Boc groups or *t*-butyl ethers and esters are removed with TFA. A particularly frustrating aspect of the tryptophan alkylation is that these *t*-butyl groups are removed upon acid hydrolysis, so that a reasonable estimate of the extent of alkylation cannot be obtained.

We believe that for most SPPS tryptophan can be used without blocking the indole ring if adequate scavengers are present during all acidic steps. We recommend indole in the deprotection reagent (see Chapter 2). Many tryptophan-containing peptides have been synthesized in good yield by this procedure. The presence of

undesired *t*-butyl tryptophan-containing peptides can usually be detected by hplc or ccd, since the alkylated tryptophan peptide should have a higher partition coefficient.

For use in labile SPPS work, α-Bpoc or Fmoc can be used. Problems associated with the use of Nps protection in tryptophan peptides were discussed above in section D.3.

n. Cysteine. The sulfhydryl group of cysteine must be blocked in peptide synthesis, and a wide range of blocking groups has been proposed for use with this amino acid. Among the several S-blocking groups available, the choice for a particular synthesis will likely depend upon the structure of the peptide being synthesized and the nature of the other blocking and protecting groups being used. For small cysteine-containing peptides, it is reasonable to use an S-blocking group which can be cleaved from the peptide simultaneously with cleavage of the peptide from the resin. If the synthesis has proceeded efficiently, relatively few "bad" peptides will be present to give by-products when the disulfides are formed. If the synthesis has not proceeded well, however, any "bad" peptide which forms a disulfide with a "good" peptide will immediately reduce the yield of "good" peptide significantly. For this reason, in the synthesis of large cysteine-containing peptides, particularly if several cysteine residues are present, there is great need for an S-blocking group which will remain stable during HF cleavage of the peptide from the resin and which can be removed selectively later after the peptide has been purified.

This concept was pioneered in solution peptide synthesis by Du Vigneaud, who introduced the S-benzyl blocking group for cysteine. The S-benzyl group **(45)** is cleaved by treatment with $Na\text{-}NH_3$. While this procedure has been used with remarkable success for a wide variety of peptides related to oxytocin and vasopressin, this procedure is not without potential harm to many peptides, and most peptide chemists would prefer to avoid its use. Following removal of the S-benzyl group, disulfide bridges are generally formed by oxidation of the peptide with air or iodine.

(45) R = $C_6H_5-CH_2-$

(46) R = $CH_3-C_6H_4-CH_2-$

(47) R = $CH_3-O-C_6H_4-CH_2-$

(48) R = $CH_3-CO-NH-CH_2-$

(49) R = $(C_6H_5)_3C-$

The acetamidomethyl group **(48)** was introduced as a modern day equivalent of S-benzyl protection. The Acm group is relatively stable to HF cleavage, and can be removed after the peptide has been purified by treatment with mercuric ion or with iodine. In the latter case, simultaneous oxidation to the disulfide occurs. Unfortunately, the Acm blocking group has not fully lived up to expectations. It is not completely stable to HF cleavage under the normal conditions. Some peptide is thus lost as free-SH peptide or as mixed disulfides during the purification procedure. Neither the mercury nor iodine treatment used for final removal of the Acm group can be considered completely innocuous, particularly with large and complex peptides. Nevertheless, it does provide an approach to maintaining protection of cysteine throughout purification of synthetic peptides.

When it is desired to remove the S-blocking group simultaneously with HF cleavage, the *p*-methylbenzyl group **(46)** appears to offer the correct stability. The *p*-methoxybenzyl group **(47)** has also been used for this purpose but it is cleaved at a significant rate by TFA, and is therefore practical for synthesis of only short peptides in conjunction with α-Boc protection.

Relatively little work has been done on synthesis of cysteine-containing peptides in more labile SPPS systems. Based on limited data, it would seem that the S-trityl group **(49)** would

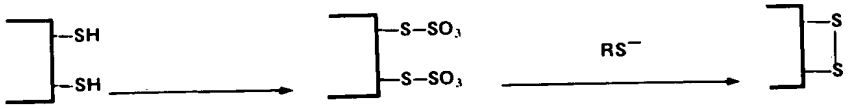
have about the right stability for this purpose. It should be removed by TFA simultaneously with cleavage of the peptide from the resin. The Acm group should also be better here than when HF is used for cleavage of the peptide from the resin.

Oxidative formation of disulfide bridges from reduced cysteine-containing peptides is still fraught with many problems. Most evidence comes from work with peptides related to oxytocin and vasopressin. With those peptides, where a single intramolecular disulfide bridge is to be formed, the peptide is customarily dissolved in very dilute aqueous solution, the pH is raised to 7 and the solution is stirred while being exposed to air. It seems to be important that the oxidation be done in a slow and controlled manner; yields are reported to be lower if air is bubbled through the solution (S. Sakakibara, personal communication). Catalysis of the oxidation by trace amounts of iron or copper does not seem to improve yields. The neurohypophyseal peptides can be oxidatively cyclized by this procedure in good yield. With other peptides where the disulfide ring is of a different size or the sequence is not related to naturally-occurring peptides, ring closure may be much more difficult and may proceed in very low yield.

sulfhydryl compound such as mercaptoethanol. This procedure has been reported to give good results with proinsulin.[34,35] In any case, it may be difficult to find just the right conditions for cyclization of the peptide to the desired disulfide form.

o. Methionine. Methionine is normally used in SPPS without specific protection on the thioether. As was mentioned above, Boc-methionine cannot be attached directly to chloromethyl resin, due to alkylation of the methionine to the sulfonium compound; rather hydroxymethyl resins should be used. Alkylation by carbonium ions can occur during HF cleavage of methionine-containing peptides from the resin, and adequate scavengers must be present to minimize this problem.

Spontaneous oxidation of methionine residues to the sulfoxide does not seem to be a serious problem in SPPS. In some cases previous oxidation to the sulfoxide has been used as a method to block methionine side chains and protect them from alkylation. Methionine can be readily oxidized to the sulfoxide by treatment with hydrogen peroxide in glacial acetic acid; this procedure is also effective for methionine-containing peptides. If the sulfoxide is used deliberately in synthesis (or if it is formed unintentionally during manipulations), the sulfoxide-containing pepide can be reduced to the desired methionine peptide by treatment with mercaptoethanol or other thiol reagents.[36] Alternatively, the methionine sulfoxide can be reduced simultaneously with HF cleavage by inclusion of 1% 2-mercaptopyridine in the cleavage mixture. This last procedure, however, does not keep the methionine protected against alkylation throughout the HF cleavage. If alkylation seems to be a



(50)

FIGURE 1-16.
Protection of Sulfhydryl Groups as the Sulfonates.

Purification and oxidation of larger peptides containing several cysteine residues is much more complex. If a cysteine blocking group is used in synthesis which is removed along with cleavage of the peptide from the resin, the -SH groups should be blocked again during purification of the peptide. Conversion of thiols to S-sulfonates (see Figure 1-16) is a practical approach to this problem. After purification of the peptide-sulfonate **(50)** it is converted to the desired sulfide by disulfide interchange with a

serious problem, the peptide can be cleaved and deblocked in the usual way with HF-anisole and then treated with HF-2-mercaptopyridine to reduce the sulfoxide in a separate step.

The biological activity of methionine-containing peptides frequently changes dramatically upon oxidation of methionine to the sulfoxide. It is thus essential to be able to know precisely the amount of sulfoxide contained within such peptides. The methanesulfonic acid hydrolysis procedure described in Chapter 2 is useful for this determination; HCl hydrolysis reconverts methionine sulfoxide largely to methionine. For a further discussion of problems with methionine peptides, see sections K and M, below.

F. THE DEPROTECTION STEP

1. Acidolysis

Acidolysis of Boc groups with TFA in DCM or anhydrous HCl in dioxane is the usual procedure in the classical SPPS system. The originally used reagent of HCl in glacial acetic acid is little used now because it does not swell the resin and may cause problems with incomplete deprotection. A 25% solution of TFA in DCM will deprotect most Boc groups in 30 minutes at room temperature. It is important that the peptide-resin be pre-washed with this same reagent in order to minimize dilution of the reagent by solvent retained in the resin beads. Such dilution may lead to incomplete deprotection. TFA can also be used in chloroform solution for removal of Boc groups; this reagent deprotects peptides at about half the rate of the corresponding concentration of TFA in DCM. Some peptide chemists prefer to use a higher concentration of TFA (for example, 50%) for a shorter period of time. The TFA deprotection reagent has been widely used, principally because of the great convenience in preparation and in spite of the high cost of TFA. Its use is not without problems, however. Commercially available TFA, even reagent grade material, may contain aldehydes which are surely harmful for tryptophan-containing peptides and may be harmful for any peptides. For all peptide synthesis we recommend storage of the TFA reagent with a scavenger such as indole overnight before the reagent is used for deprotection.

Use of the TFA deprotection reagent can also lead to unwanted premature termination of peptide chains on the resin by trifluoroacetylation.[37] Proline residues are especially susceptible to this termination reaction, apparently due to the unusual basicity of N-terminal proline residues (pK = 9.5, vs. 8 for other residues). Even though the amino group of proline is secondary, the ring structure apparently minimizes any steric hindrance and makes the amino group easily available for reaction. For a further discussion of termination by trifluoroacetylation, see section K below.

In a recent paper, Lukas *et al.*[38] reported that the actual deprotecting agent for acidolytic cleavage of Boc groups is not the TFA molecular species but rather a protonated form of TFA which exists in the usual deprotection solutions in extremely low concentration. They reported that if TFA was protonated by addition of a small amount of methanesulfonic acid the concentration of this protonated species necessary for deprotection of Boc groups was very low. For example, whereas 25% TFA in DCM is 3.3 *M*, they obtained rapid deprotection of Boc groups with 0.01 *M* MSA and 0.1 *M* TFA in DCM. In a continuous flow SPPS system, this solution was pumped through the resin for 5 minutes, using 5 equivalents of the protonated species per equivalent of Boc group on the peptide. This reagent appears to be considerably more vigorous than the usual TFA deprotection reagent, and it must be used with caution. It is also not clear at this time if these investigators are really dealing with a reagent significantly different from one they might have made using MSA alone.

A 4.0 *M* solution of HCl in dioxane has been used extensively in SPPS for deprotection of Boc groups. It has not been used much in recent years, however, because of the additional work involved in making the reagent compared to the ease of use of TFA. The facile oxidation of dioxane by air is also a problem; it is difficult to obtain peroxide-free solvent. Even the apparently simple method for removal of peroxides by

passing dioxane through a column of alumina is not actually so simple, since it can be difficult to find appropriate grades of alumina which are effective for this purpose. Dioxane is reported to have a significant toxicity, and this hazard must be taken into consideration when using it. There are, however, advantages associated with use of the HCl-dioxane deprotection reagent. In the first place, it appears to be less aggressive than the TFA-DCM reagent, and loss of peptide from the resin is slower. In the second place, even when peptide is lost, the internal trifluoroacetylation mechanism depicted in Figure 1-17 cannot take place, since no TFA is present. One additional point concerning use of HCl-dioxane for deprotection is that triethylamine hydrochloride is not very soluble in DCM; chloroform should be used for the neutralization solvent.

Reagents for acidolytic deprotection in labile SPPS systems can be prepared from dilute solutions of TFA in DCM, as discussed in Chapter 2.

2. Other Methods of Deprotection

When Fmoc amino acids are used in SPPS, the protecting group is normally removed by treating the peptide-resin with a mixture of equal parts of piperidine and DMF. Several variations have been proposed in the actual reagent used for Fmoc deprotection. The use of dimethylacetamide is reported to have some advantages, but the very high cost of this solvent makes its use prohibitive. As was mentioned earlier, use of this strong piperidine solution is not compatible with peptide-resin stability in the standard Merrifield SPPS system; acid-labile peptide-resin links, such as that in the ether resin **(3)** must be used.

Reagents for removal of the Nps and Dts groups were discussed in section D, above, in the discussion of those protecting groups.

There is still a need for development of new and improved deprotection reagents. The "push-pull" mechanism proposed by Kiso *et al.*[39] should be investigated for deprotection of Boc groups.

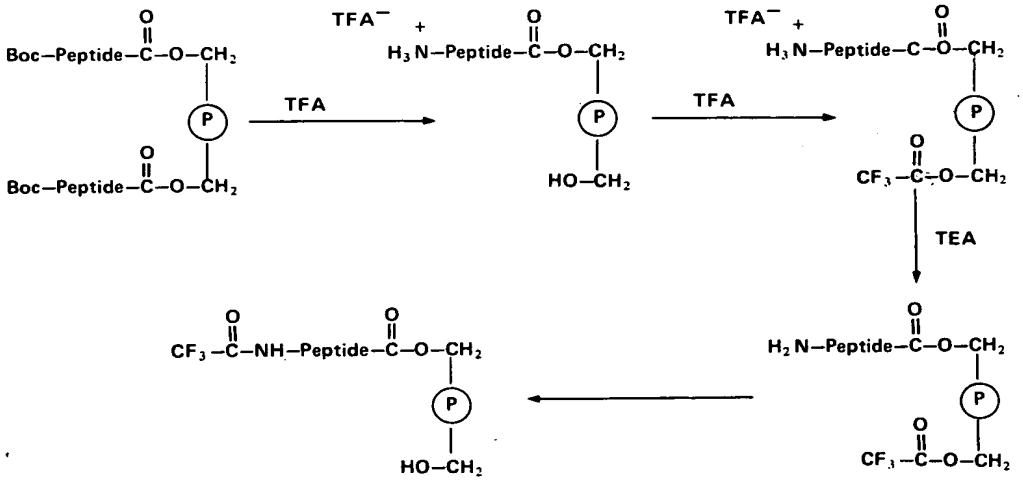


FIGURE 1-17.
A Mechanism for Termination of Peptides by Trifluoroacetylation.

G. THE NEUTRALIZATION STEP

When acidolysis is used to deprotect peptides in SPPS, the newly formed amino group is left as a salt of the deprotecting acid. This salt must be neutralized to yield the free amino group before the coupling reaction can be carried out. The neutralization is usually carried out by treating the peptide-resin with a tertiary amine. A 10% solution of triethyl amine in DCM, CHL or DMF has traditionally been used as the neutralization reagent. TEA should give rapid and complete neutralization, particularly if two washes with the reagent are used. Although earlier practice specified a 5- or 10- minute treatment with TEA, this is probably neither necessary nor desirable. TEA in DCM is the standard neutralization reagent when TFA is used for deprotection; when HCl is used for deprotection, TEA in CHL should be used. The use of DMF as a neutralization solvent is not recommended.

When a Boc-amino acid is attached to chloromethyl resin, TEA can react with chloromethyl groups on the polymer to form quaternary ammonium groups on the resin. These are undesirable, since they act as anion exchange groups. Moreover, this same reaction can take place slowly at every neutralization step during the synthesis if excess chloromethyl groups are present on the resin. For this reason, we recommend use of hydroxymethyl resin as starting material for standard SPPS. The sterically hindered diisopropylethylamine has been recommended over TEA because it reacts more slowly with resin-bound chloromethyl groups. If hydroxymethyl resin is used, the very significant added cost of using DIEA is eliminated. Fresh reagent grade TEA is generally satisfactory, unless a long peptide is being synthesized, when additional precautions may be justified.

TEA also reacts with the halogenated solvents used in neutralization. The quaternary compounds thus formed are probably not harmful, although some concern has been raised by the presumed transient carbene intermediate in these reactions. In any case, this reaction will decrease the concentration of tertiary amine available for neutralization, and for this reason, the neutralization reagent should be prepared anew at frequent intervals. This reaction can also be minimized by using DIEA as the neutralizing amine.

Whatever method of neutralization is used, the operator should proceed rapidly from the neutralization through the washes to the coupling reaction. Several kinds of harmful side reactions can occur if the coupling reaction is delayed. If there are residual chloromethyl groups on the resin, the peptide free amino group may react with these halogens to tie the peptide covalently to the resin. Any peptide chain which undergoes such a reaction is obviously lost completely. When a dipeptide-resin has been neutralized, the amino group may attack the ester anchoring the peptide to the resin, splitting the dipeptide from the resin as a diketopiperazine (Figure 1-18). This reaction is particularly bad if the dipeptide contains proline or other secondary amino acids. N-terminal glutamine peptides are also prone to cyclize to the pyroglutamyl form (see Figure 1-14). The trifluoroacetylation reaction shown in Figure 1-17 can also take place at this point. All

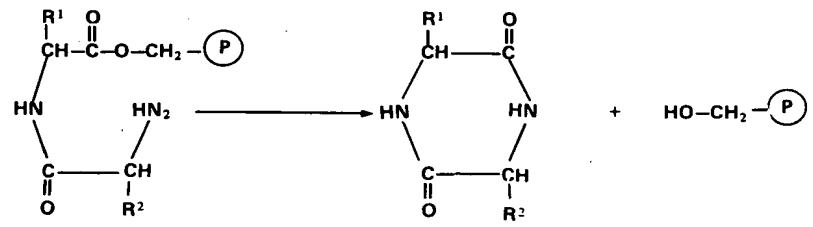


FIGURE 1-18.
Cleavage of Dipeptides by Diketopiperazine Formation.

of these undesired reactions can be expected to take place more rapidly when DMF is used as solvent, since it promotes nucleophilic reactions. The diketopiperazine formation and glutamine cyclization appear to be catalyzed by weak acids. When the deprotection reagent contains HOAc or TFA and DCC will be used in the coupling reaction, it is particularly important that the neutralization be thorough to avoid such catalysis or termination of the peptide chain by activation of these acids. These problems are discussed further in section K, below.

H. THE COUPLING REACTION

Attaining quantitative coupling reactions is probably the greatest single technical difficulty in SPPS at the present time. The coupling agent originally used by Merrifield was DCC, and it remains the most widely used. However, inadequacies of DCC-mediated coupling reactions in many peptide sequences have led to investigation of other coupling procedures. Some characteristics of an ideal coupling agent are general applicability, ease of use (particularly with automatic synthesizers), rapidity and efficiency of coupling reactions, and freedom from racemization and other side reactions.

In general, it may not be wise to prolong coupling reactions indefinitely to achieve completeness in those cases where the reaction is slow. Racemization clearly becomes a greater problem in prolonged, slow coupling reactions. In most cases, if monitoring shows lack of complete coupling after several hours, it is preferable to stop the reaction and recouple. An exception to this rule would be the case of amino acids having substitution on the α-carbon or the amino group. These frequently couple very slowly and will not be expected to racemize readily. Recoupling procedures should always include a repetition of the neutralization step.

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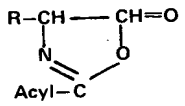


I. Carbodiimides

Coupling reactions mediated by DCC are, in most cases, quite rapid due to the high degree of activation afforded by the O-acyl isourea **(51)**. It is this high degree of activation which leads to formation of azlactones **(55)** when a peptide carboxyl group is activated with DCC. This causes problems in segment condensation procedures in SPPS. On the other hand, when a single amino acid bearing an N-urethane protecting group is activated by DCC this is not generally a serious problem. This latter is a major point in favor of the stepwise SPPS approach.

In DCC-mediated coupling reactions (see Figure 1-19), the isourea intermediate **(51)** rearranges spontaneously to the N-acyl urea **(52)**. The acyl urea is not reactive and represents undesirable loss of activated Boc-amino acid from the coupling reaction. The rate of this undesirable rearrangement is increased in polar aprotic solvents such as DMF, and therefore the amount of DMF present in DCC-mediated coupling reactions should be kept to the minimum. The presence of dissolved salts in the solution increases the ionic strength of the medium and accelerates the rearrangement. The rate of rearrangement is also dependent upon the nature of the amino acid. Since the rearrangement is intramolecular, the rate does not depend upon concentration, but on the factors already mentioned. Since the rate of the desired coupling reaction is increased with increasing concentration, DCC-mediated coupling reactions are usually carried out in the minimum amount of solvent needed to dissolve the Boc-amino acid and swell the resin. Loss of activated intermediate by this rearrangement, as well as the need to maintain a good rate of coupling until the last of the peptide is elongated, make the use of large excesses of Boc-amino acid and DCC mandatory in SPPS. The basic routine procedure recommended in this book calls for use of a 2.5-fold excess of Boc-amino acid and DCC over peptide amino groups. Many SPPS coupling reactions go to completion within 10 or 15 minutes under these conditions. When coupling reactions are being monitored, attainment of a

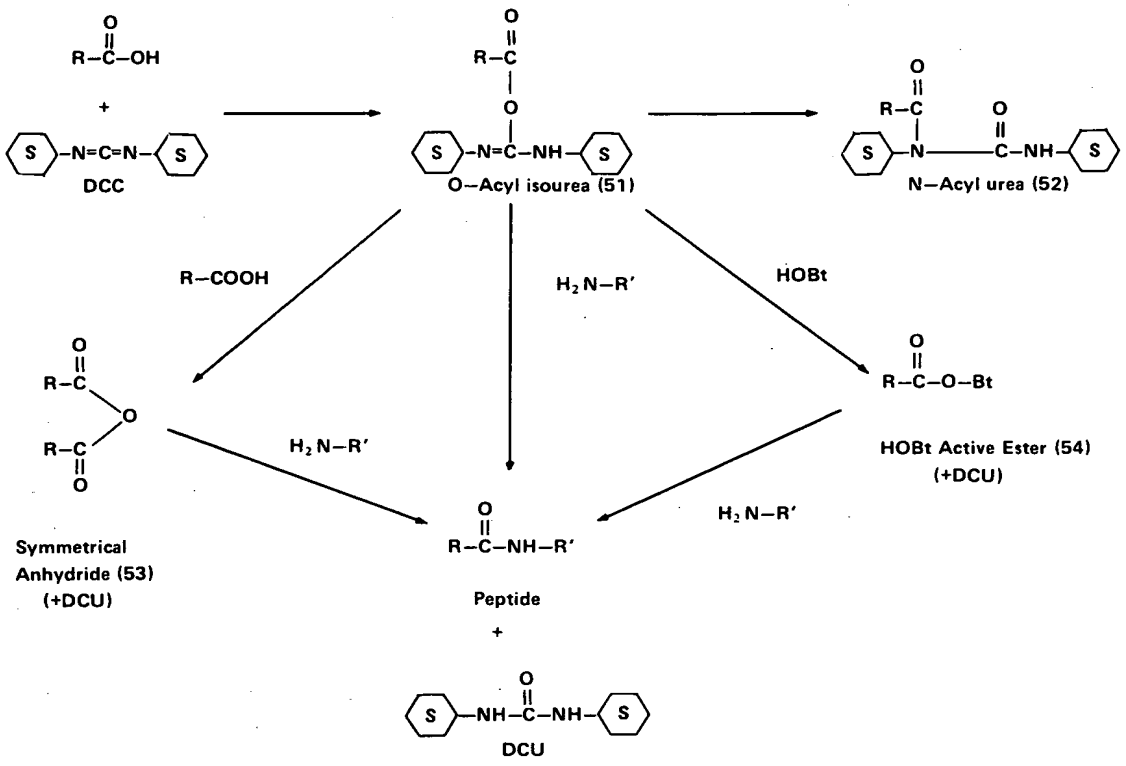


FIGURE 1-19.
The DCC Coupling Reaction.

negative test for free amino groups is the signal for the operator to proceed to the next residue. In automatic synthesis, coupling reactions are usually extended a standard two hours for convenience in programming. Although it has not been specifically recommended and described in this book, the "adsorption coupling" method[40] has been reported to give increased efficiency of use of the Boc-amino acid, and may be useful in cases where the amino acid is extremely valuable or scarce.

As indicated in Figure 1-19, the use of two equivalents of Boc-amino acid with 1 equivalent of DCC leads to formation of the Boc-amino acid symmetrical anhydride **(53)**. There is evidence to indicate that the symmetrical anhydride is an actual intermediate in many SPPS coupling reactions, even when equimolar amounts of Boc-amino acid and DCC are used. In solution synthesis, on the other hand, the O-acyl isourea **(51)** may react directly with the amine component of the peptide synthetic mixture. Some investigators feel it is wise to favor intermediate anhydride formation by use of the 2:1 ratio of Boc-amino acid:DCC, but if the demonstrated advantages of anhydride coupling reactions are to be exploited to their fullest, it is more reasonable to use a pre-formed symmetrical anhydride, as discussed in the next section.

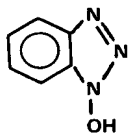
In most SPPS coupling reactions the Boc-amino acid solution has been added to the peptide-resin and mixed for five minutes before addition of DCC in order to allow the amino acid to penetrate the resin beads. This procedure has been found to be definitely harmful when the third residue is being added to the resin (loss of dipeptide as diketopiperazine) and when a Boc-amino acid is being coupled to an N-terminal

glutamine peptide-resin (cyclization to pyroglutamyl peptide). Both these problems are discussed below. On the other hand, the known reaction of DCC with amines (the reason the practice of later addition of DCC was originally adopted) does not seem to be a problem with peptide-resins. For these reasons, the pre-mixing of the amino acid derivative with the resin can be minimized in all cases, and surely eliminated in those cases where problems are anticipated.

The water-soluble carbodiimide EDC, which is also freely soluble in DMF and moderately soluble in DCM (saturated at about 0.25 *M*), has been used to a limited extent in SPPS, and some chemists believe it offers advantages of few side reactions. Disproportionation of such unsymmetrical carbodiimides may lead to some problems.

Diisopropylcarbodiimide has recently become available commercially (Aldrich), and is being used by some peptide chemists. Since it is a liquid, it can be measured by pipetting. This is a considerable improvement over the difficulty of chipping the waxy dicyclohexylcarbodiimide out of a bottle. It is also said to give byproducts (diisopropylurea and diisopropyl-N-acylureas) more soluble than those with DCC.

(56)



As described earlier, Boc-amino acid-*p*-nitrophenyl active esters can be synthesized in solution by the use of DCC. As indicated in Figure 1-19, an active ester can be synthesized *in situ* in the coupling mixture by addition of a suitable negatively substituted alcohol to the coupling mixture. The alcohol most commonly used for this purpose is 1-hydroxybenzotriazole **(56)**. Coupling reactions in certain difficult sequences have been shown to proceed demonstrably faster by this modification of the DCC coupling reaction. Better results are often obtained by use of preformed HOBt active esters, as discussed in section 3, below. As mentioned earlier, HOBt should not be added to coupling reactions when the peptide contains tosyl histidine, since the tosyl group is removed by HOBt.

2. Symmetrical Anhydrides

Preformed Boc-amino acid symmetrical anhydrides have been found to give best results of all methods tried in many difficult sequences. Since Boc-amino acid symmetrical anhydrides are usually prepared immediately before use, their application to automatic synthesizers creates some inconvenience. In manual SPPS, on the other hand, the slight additional work is of little consequence. One might think that automatic operation could be carried out by storing the anhydride solution in a chilled vessel, but this may not be practical, since these solutions may precipitate or gel at the necessary concentration of reagents. Another advantage of anhydride coupling reactions is that peptide chains cannot be terminated by DCC-activation of any residual TFA or HOAc remaining in the resin at the coupling step. Symmetrical anhydrides should NOT be used for coupling Boc-Arg(Tos); it has been found to cause undesired insertion reactions. That is, more than one residue of arginine may be added to the peptide in a single coupling step.

Although Boc-amino acid symmetrical anhydrides frequently give dramatically increased reaction rates in difficult SPPS coupling reactions, especially when an equivalent of tertiary amine is added to the mixture, one should not expect that anhydrides will always give the best coupling yields. It is by no means always possible to predict the relative rate of the coupling reaction in SPPS. One can, of course, anticipate that coupling the sterically hindered amino acids (such as valine, isoleucine or threonine) to a peptide-resin in which the N-terminal residue is sterically hindered will be slow. However, there are other occasions when coupling reactions will not go to completion even upon repeated application of symmetrical anhydrides and when there is no obvious steric reason for the coupling reaction to be slow. These problems are clearly sequence dependent and apparently represent some interaction of the amino terminus of the

peptide chain on the resin either with itself or the polymer matrix. In some cases, a Boc-amino acid active ester has been found to give complete coupling even though the anhydride did not.

In contrast to the situation with Boc-amino acid symmetrical anhydrides, the symmetrical anhydrides of Fmoc-amino acids are reported to be stable, and can be synthesized in advance and stored until they are needed.

3. Active Esters

Use of *p*-nitrophenyl active esters of asparagine and glutamine was discussed in section E, above. These active esters have not been used very widely in SPPS because their low reactivity necessitates use of large excesses of active ester and very long reaction times. Moreover, the DMF solvent used for active ester coupling reactions may tend to promote racemization.

Following the demonstration of dramatic improvement in solution peptide synthesis by use of HOBt active esters, either preformed or generated *in situ*, this procedure was applied to SPPS, again with good results. The use of *in situ* generated HOBt esters has been applied particularly widely to coupling of Boc-asparagine and Boc-glutamine without protection on the omega-amide. The full advantages of HOBt active esters in SPPS can usually be attained only by preforming the active esters in a separate reaction before addition to the peptide-resin.

N-Hydroxysuccinimide active esters have been much used in solution peptide synthesis. Although there have been isolated reports of favorable results from the use of Boc-amino acid-OSu procedures in SPPS, use of HOSu active esters, either preformed or formed *in situ* by addition of HOSu to the DCC-mediated coupling mixture, has not appeared to give sufficiently good results that it can be recommended generally.

As mentioned earlier, active ester coupling reactions can be accelerated by addition of an equivalent of acetic acid to the coupling mixture. This is usually done after the active ester has been allowed to couple for some time without an additive.

4. Other Coupling Agents

In addition to these three major procedures for formation of peptide bonds in SPPS, other coupling reactions have been used. These are listed and described in the review of Barany and Merrifield.[1] In general, these have not been used extensively enough as yet that one can make general statements about their applicability for routine work. In some special cases they may be helpful. For example, the azide coupling method has been found to be effective for segment condensation reactions in SPPS.

I. MONITORING SOLID PHASE SYNTHESIS

When the standard procedures of SPPS are used for synthesis of short, simple peptides, particularly when automatic synthesizers are used, it is often practical to run through the entire sequence automatically without monitoring the various steps of the synthesis. Under these circumstances the synthesis will usually be found to have proceeded quite satisfactorily, yielding the desired peptide as the major product. Side reactions leading to truncated or deletion sequences may occur only to a slight extent, and in any event the by-products resulting from such side reactions may be readily separated from the desired product by routine purification procedures. If such syntheses are routinely done on a rather small scale, such as 0.4 mmole, the expenditure of time and reagents is of less consequence than the additional time necessary to monitor the various reactions. The entire sequence can be synthesized automatically and the crude product examined, preferably by paper or thin layer electrophoresis, for the presence of the desired product. In many cases, single coupling reactions yield quite satisfactory results. In other cases, where there is some prior indication that a particular step or region of the peptide might not proceed satisfactorily, automatic recoupling reactions can be included. If results of this preliminary run-through are not satisfactory, the information obtained from the products can be used to direct resynthesis. On the other hand, when long peptides are being synthesized or when

there is good reason to suspect serious problems, each step of the synthesis should be monitored. Monitoring adds little to the time and effort involved in manual SPPS, and can be done routinely.

Monitoring procedures may be either qualitative or quantitative, and they may be used in a discrete fashion (to indicate the status of the peptide-resin at a particular moment) or less frequently in a continuous fashion (to follow the course of a reaction as it is taking place). Qualitative, discrete monitoring can be done rapidly and simply, and the speed of such procedures makes them, in a sense, almost like continuous monitoring procedures. In these procedures, a small aliquot of peptide-resin is removed from the reactor and examined with a suitable reagent. Application of discrete, quantitative procedures to an aliquot of peptide-resin is inherently slower, since the sample of resin must be dried and weighed accurately before the analytical procedure is applied. The quantitative, discrete monitoring procedures can also be applied to the entire batch of peptide-resin in the synthesis; the picric acid titration of Merrifield and the perchloric acid titration procedure of Brunfeldt are examples. These procedures in which the entire batch of peptide-resin is titrated can probably not be recommended for general use. The reagents used may cause some harm to the peptide being synthesized, particularly if side-chain functions and their blocking groups are not completely stable. If these procedures are used, one will probably wish to perform a preliminary run-through of the complete synthesis to determine optimal conditions for the various steps and then repeat the synthesis with a fresh batch of amino acid-resin, utilizing the results of the preliminary run. Continuous monitoring of SPPS reactions has been done by following uv absorbance of the reaction solution. This procedure requires apparatus suitable for continuous circulation of filtered reaction mixture through a spectrophotometer cell. This has been done rather simply in conjunction with the centrifugal solid phase reactor designed and used by Birr;[41] it has also been done by Rudinger[42] by using a pump to circulate the filtered solution from a conventional reactor through the cell of a spectrophotometer. Other investigators have also used similar procedures.

For the most part, precision of the available SPPS monitoring procedures leaves much to be desired. The principal exception to this statement is the monitoring of peptide-resins for completion of coupling by measuring remaining amino groups on the resin. Since complete coupling leads to a null point, this determination can be done with good sensitivity and precision; even qualitative procedures can give very useful results in this case. Determination of the endpoint in deprotection or coupling reactions by continuous flow spectrophotometry or monitoring deprotection by discrete titration methods leaves much to be desired, since the endpoint is approached asymptotically. Use of Edman sequencing of peptide-resins for detection of "deletion sequences" is discussed in section K, below.

1. Monitoring Deprotection

A rapid, qualitative indication of the progress of deprotection of peptide-resin can be obtained by application of the Kaiser ninhydrin test to an aliquot of the resin. Deprotected peptide-resins should give an intense blue color with the reagent. N-terminal proline peptide-resins give a brown color. There have been a few isolated instances of deprotected peptide-resins which do not give a positive reaction in the Kaiser test; this is usually due to the presence of some unnatural amino acid which does not react well with ninhydrin. Normally, the qualitative Kaiser test cannot distinguish relative degrees of deprotection, particularly toward the end of normal deprotection reactions. It may be useful in some cases where monitoring of completeness of coupling gives ambiguous results or one may wish to examine a peptide to see if chain termination has occurred.

Any of the quantitative monitoring procedures can give a better estimate of completion of deprotection. Titration of the entire batch or an aliquot of the resin by the perchloric acid[43] or picric acid[44] methods or application of the quantitative ninhydrin procedure[45] to an aliquot of resin can be used. Whereas the titration methods are basically non-destructive, the ninhydrin pro-

cedure is destructive, and should be used on as small an aliquot of resin as is practicable.

2. Monitoring Coupling

Monitoring procedures are most often applied to the coupling reaction in SPPS. Not only is the coupling reaction most likely to be the source of incomplete reactions when standard SPPS procedures are used, but the inherent nature of coupling monitoring procedures leads to more precise results. Any method which examines the peptide-resin for absence of remaining free amino groups can, in principle, yield useful information on completeness of coupling. The Kaiser reaction is doubtless the most frequently used monitoring procedure for this purpose. It is rapid and simple and is applicable to all N-terminal residues. This last point gives the Kaiser test a distinct advantage over the Fluorescamine and TNBS tests, which do not respond to N-terminal proline residues. While the general criticism of the qualitative coupling monitoring procedures is that they lack sensitivity to determine the last few tenths of a percent of remaining amino groups, the Fluorescamine reaction is perhaps too sensitive for general practical use. Spurious fluorescence can be introduced in various ways, and the presence of fluorescent groups in the peptide (for example, Asn(Xan) or Gln(Xan) groups) make this procedure inapplicable.

The quantitative monitoring procedures which depend upon titration of basic groups on the resin do not uniformly approach a zero endpoint at completion of coupling, since some groups in the peptide-resin may yield a considerable background level of titratable groups which may even increase as the peptide chain grows. In contrast, the quantitative ninhydrin reaction may be most practical for monitoring of coupling reactions. Unfortunately, the quantitive ninhydrin procedure, which depends upon production of a soluble dye which is extracted from the resin, does not respond to N-terminal proline peptide-resins. In this case, the pigment remains attached to the resin and cannot be extracted for quantitative measurement.

Whatever monitoring reaction is applied for estimation of completeness of coupling, it is critically important that the peptide-resin used in the test be thoroughly washed to remove substances which may give a false positive indication.

3. Monitoring Chain Termination

The growing peptide chains may be inadvertently terminated by trifluoroacetylation if TFA is used for deprotection, by cyclization of N-terminal glutamine residues to pyroglutamyl peptides or in certain cases by transfer of terminating groups from side-chain functions. If a significant percentage of the growing chains has been terminated, application of even a qualitative monitoring procedure may reveal a less than normal intensity of color after deprotection. This has often been observed by use of the Kaiser test, when the deprotected peptide-resin no longer gives an intense blue color and a dark blue solution. Accurate determination of the degree of chain termination will require application of one of the quantitative monitoring procedures. Amino acid analysis of hydrolysates of peptide-resins is very useful for detection of termination; a homogeneous peptide-resin will show expected ratios of amino acids throughout the length of the peptide chain. When termination has occurred, the C-terminal residues will be present in higher ratios. Since protecting groups have been found to be unusually resistant to the usual deprotection reagents in certain cases, one should keep in mind that a decrease in available amino groups after deprotection may be due to either permanent chain termination or incomplete removal of the α-blocking group. The latter case can be detected by repetition of the deprotection step and again applying the quantitative monitoring procedure. If available amino groups have increased, incomplete deprotection is indicated.

4. Monitoring Loss of Peptide from the Resin

The various means by which peptide chains may be lost from the polymer support during synthetic operations have been discussed in preceding sections. Such loss is indistinguishable from permanent chain termination when quantitative monitoring procedures are applied. In the synthesis of short peptides, such loss is usually

not significant except in the case of loss of dipeptides by cyclization to diketopiperazines (see section K, below). In the synthesis of long peptides, however, such loss of peptide chains may become serious, due to the repetition of the deprotection step. This is a serious problem when the standard Merrifield resin is used in the classical SPPS system, and is less of a problem if Pam resin is used with Boc-amino acids, if a very labile α-blocking group is used, or if an orthogonal system is used. The situation is particularly confusing if chain termination is occurring at the same time. Even amino acid analysis of hydrolysates of peptide-resins is frequently confusing, particularly if the C-terminal residue appears several times in the peptide sequence. As the peptide-resin gains weight with the growing peptide chain, the degree of substitution of the original C-terminal residue, on a weight basis, will continually decrease. While the dilution factor due to weight gain can be calculated from the actual dry weight of peptide-resin at any point, this procedure is confounded by repeated removal of aliquots of peptide-resin for monitoring purposes. A practical approach[46] to determination of peptide loss from the resin in this situation lies in the use of Pam resin in conjunction with an internal reference amino acid. The internal reference amino acid, one which does not appear in the peptide being synthesized, is attached to the polymer by a bond which will be completely stable to all procedures of peptide synthesis. Total hydrolysis of the peptide-resin with HCl-propionic acid, however, will remove the reference from the polymer as the entire peptide is being hydrolyzed. If the reference amino acid is an unnatural one which has a separate and distinct position on the amino acid analyzer, a generally useful resin can be synthesized and used for synthesis of a variety of large peptides. A practical system is outlined in Figure 1-20. Aminomethyl resin, having a higher degree of amine substitution than is desired for the C-terminal residue, is acylated in a DCC-mediated coupling reaction with a mixture of the Boc-amino acid-Pam link **(5)** (see Figure 1-6) and the acetyl reference amino acid. The product **(57)** contains the acetyl reference amino acid linked permanently to the resin by a bond which will not be cleaved even by HF. Moreover, the acetyl

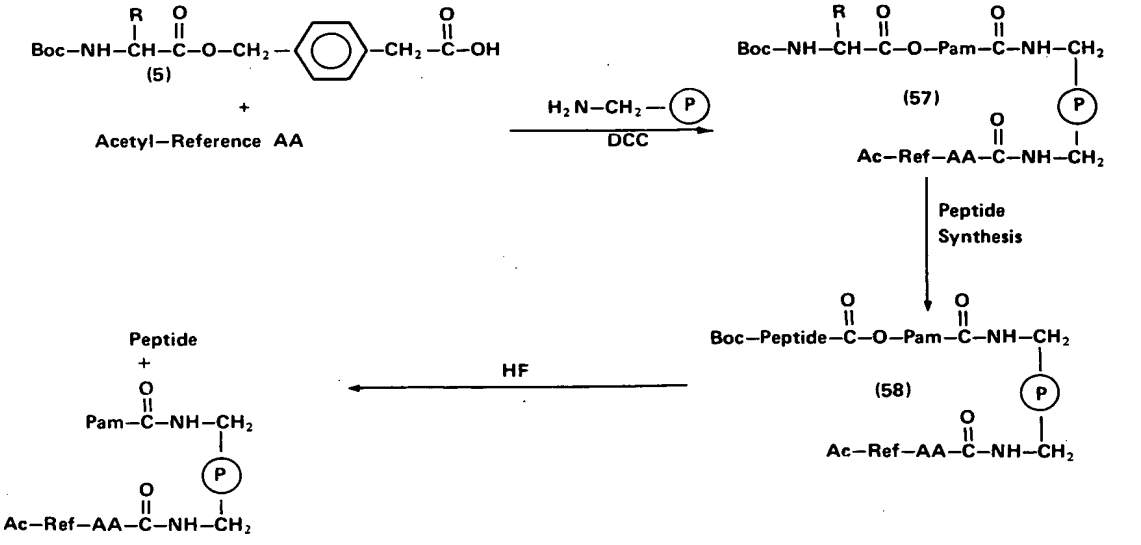


FIGURE 1-20.
Use of an Internal Reference Amino Acid With Pam Resin.

group will not be removed by any of the procedures of SPPS. Therefore, peptide chains will not grow onto this reference residue. SPPS procedures are then used to grow the peptide on the amino acid attached to the Pam handle **(58)**. At any point in the synthesis, accurate determination of the substitution of the reference amino acid on the polymer (following acid hydrolysis) gives an accurate measure of the percentage weight gain of the peptide-resin and permits calculation of the dilution factor, no matter how much has been removed for monitoring. A clear differentiation can then be made between peptide chain termination and loss of peptide from the resin. At the end of the synthesis, HF cleavage will yield the peptide without any contamination by the Pam link or the reference amino acid. This approach has been used in several cases, and has permitted clarification of otherwise confusing situations in analysis of the progress of synthesis of long peptides.

J. CLEAVAGE OF PEPTIDES FROM THE RESIN

Much of the versatility of the Merrifield system of SPPS lies in the fact that different reagents can be used to cleave the finished peptide from the resin, thus yielding a variety of final products. Several options for cleavage are indicated in Figure 1-2.

1. Acidolysis

The most popular reagent for cleavage of peptides from the resin at the end of the synthesis is anhydrous liquid HF. Of all the cleavage procedures used until now, this appears to be the most versatile and least harmful to a wide variety of peptides. Some specific problems are discussed in section K, below. HF is a toxic, corrosive gas (boiling point 19°), and it must always be used in an adequate fume hood. Since it attacks glass very rapidly, with an exothermic reaction, all equipment for handling HF must be made exclusively of plastic or non-corrosive metal. The commercially available plastic vacuum line for handling HF allows all of the operations necessary for successful SPPS cleavage reactions to be carried out without any hazard to operators. It is important that all persons using the equipment understand its operation thoroughly and follow explicit instructions carefully. This commercial apparatus also allows transfer of HF under vacuum; this is particularly important for removal of HF at the end of the cleavage reaction. Several side reactions have been shown to be temperature dependent and are much worse at elevated temperatures. Simpler equipment for handling HF can be constructed of heavy-walled fluorocarbon bottles, tubing and valves, but these generally do not withstand vacuum and the operator must use a stream of nitrogen for removing HF. A consequence is that the finished peptide is exposed to HF at elevated temperature for an extended period of time.

HF cleavage is generally done at 0° for 30 minutes; these conditions will generally cleave the peptide effectively from the resin and remove all side-chain blocking groups. Some side reactions which occur during cleavage have been found to be temperature dependent, notably acylation of anisole by glutamic acid side-chain carboxyl groups and the N-to-O shift which can occur at serine and threonine residues. Since many of the side-chain blocking groups are removed at significantly lower temperatures by HF, some investigators prefer to do cleavage at a lower temperature, or at least a preliminary cleavage at a lower temperature followed by cleavage at 0°. This may give a significant improvement in results, particularly if the peptide is large and contains many residues with susceptible side-chains. In one recent synthesis[33] the peptide-resin was treated with HF at -10° for 30 minutes and then at 0° for 30 minutes.

When side-chain blocking groups are cleaved by acidolysis, reactive carbonium (or nitronium) ions are formed which can attack easily alkylatable residues in the peptide. Benzyl and *t*-butyl carbonium ions, for example, can readily alkylate methionine, cysteine, tyrosine and tryptophan residues. These destructive alkylations can be largely prevented if a large excess of a suitable nucleophilic scavenger is included in the HF reaction mixture. Anisole has been most frequently used for this purpose, but other nucleophiles may be even more effective. Resorcinol, thioanisole, dimethylsulfide, ethylmethyl-

sulfide, methionine, 1,2-dithioethane, and indole have all been used for this purpose, with favorable results. Many investigators use a mixture of several scavengers simultaneously, with apparently improved results in the case of very sensitive peptides.

In some recent work peptide-resins have been cleaved successfully by use of a low concentration of HF in a large amount of anisole or other nucleophilic scavenger reagent (such as a sulfide) in a manner analogous to the "push-pull" deprotection procedure of Kiso.[39] Most recently the Merrifield group[8a] has found that many of the problems encountered in use of the usual HF cleavage can be overcome if a new "low HF" procedure is used. If the cleavage mixture consists of an equimolar mixture of HF and dimethyl sulfide (1:3 by volume) the cleavage mechanism changes from the usual S_N1 (where carbonium and nitronium ions are produced) to S_N2, where the high concentration of dimethyl sulfide causes it to attack the initial protonated intermediate before separation of a discrete carbonium ion can occur. This new procedure prevents alkylation of tyrosine by O-benzyl or O-dichlorobenzyl groups, formation of succinimide peptides from Asp-Gly sequences and acylation of scavenger molecules by glutamyl side chains. It reduces methionine sulfoxide residues to methionine residues. Para-cresol is recommended as a scavenger in this mixture. If *p*-thiocresol is added to the cleavage mixture, formyl groups are cleaved from tryptophan residues. This "low HF" procedure does not cleave Arg(Tos), Arg(NO_2), Asp(O-cyclohexyl), Cys(Meb) and His(Dnp) groups, and may not cleave peptides from BHA resins. If the "low HF" procedure is followed by a standard HF cleavage, Arg(Tos), Arg(NO_2) and Cys(Meb) groups will be cleaved, and peptides will be more effectively cleaved from BHA resins (some may not cleave).

The original cleavage reagent introduced by Merrifield was HBr in TFA. Since HBr is not very soluble in TFA, the cleavage is performed by bubbling a constant stream of HBr through a suspension of the resin in TFA. The commercially available HBr in glacial HOAc must not be used, since it causes acetylation of serine and threonine hydroxyl groups. Trifluoroacetylation has not been shown to occur with HBr-TFA. The HBr-TFA reagent is convenient for cleavage if HF apparatus is not available. The major drawback is that arginine blocking groups are not cleaved by HBr, and must be removed later in a separate reaction if arginine is present.

Another cleavage reagent which can be used in the absence of HF apparatus is a mixture of TFMSA, TFA and DCM.[26] Like HBr, this reagent does not remove nitro or tosyl blocking groups from arginine. Moreover, it is a more powerful Friedel-Crafts catalyst than is HF, and greater problems with side reactions may be anticipated. A mixture of very effective scavengers must generally be used with this cleavage reagent. Methanesulfonic acid has also been used for cleavage of the peptide from the resin, but it is a much less vigorous reagent than any of the previously discussed acids, and generally will not be satisfactory for use in the classical SPPS system.

A complex of HF with pyridine is commercially available and has had some application as an SPPS cleavage reagent. One problem this reagent has in common with MSA and TFMSA is lack of sufficient volatility so that the reagent may be removed conveniently by evaporation under vacuum. When these reagents are used for cleavage, the peptide must either be precipitated from solution with a dry solvent such as ether, or else the acid can be diluted with a large volume of ice water. Neither of these procedures is particularly convenient.

When the ether resin **(3)** is used for SPPS, the final cleavage may be carried out with TFA-DCM. Effective scavengers must also be used in this procedure, since *t*-butyl carbonium ions are extremely reactive alkylating agents. In general, whenever acidolysis will be used for final cleavage, the peptide synthesis should be finished with a deprotection step so that the α-protecting group is removed before cleavage, thus eliminating one potential alkylating agent.

When peptides are assembled on BHA or MBHA resins, HF treatment yields the peptide amide cleanly (see Figure 1-9). As was pointed out above, the rate of this cleavage reaction is very dependent upon the nature of the C-terminal residue in the peptide. Both of these

resins have a peptide-resin bond more stable than that in the classical Merrifield SPPS system, and particularly with the BHA resin, peptides with C-terminal phenylalanine, leucine, isoleucine, or valine residues may not be cleaved at a satisfactory rate.

2. Ammonolysis and Aminolysis

The benzyl ester linking the peptide to the resin in the classical Merrifield SPPS system can be cleaved by ammonia and amines to yield peptide amides (see Figure 1-2). These reactions are quite slow, and should be carried out in solvents which swell the resin. Among others, DMF has been used for this purpose, although it may be cleaved partially under the conditions of the reaction and lead to formation of peptide dimethylamides. Ammonolysis in liquid ammonia is very slow, since this solvent does not swell the resin. A small amount of tertiary amine may improve the rate by catalyzing the reaction. Dimethylaminoethanol has been reported[47] to give satisfactory cleavage of peptide-resins, and yields the dimethylaminoethyl ester. This ester can be hydrolyzed very rapidly by treatment with mild aqueous base to yield the peptide acid.

3. Reductive Cleavage to Alcohols

Peptide alcohols (in which the C-terminal carboxyl group is reduced to an alcohol) have been found to have interesting properties, particularly in the enkephalin field. This prompted us to investigate reductive cleavage of the peptide-resin ester bond in classical Merrifield resins.[48] Reductive cleavage proceeds rapidly when the peptide-resin is treated with lithium borohydride in tetrahydrofuran. If the peptide contains glutamic and aspartic acids protected as esters, these side chains will also be reduced to the alcohols.

4. Other Cleavage Reactions

Peptide esters can be formed by transesterification of the peptide from the Merrifield resin, using a tertiary amine as catalyst. Reasonable rates are attained only if the alcohol is simple. Side-chain blocking groups are not removed in this procedure and must be cleaved by subsequent treatment with an appropriate reagent, such as HF. One must remember that glutamic and aspartic side chains will also be converted to the same ester as the C-terminal residue; this may cause them to be stable to the subsequent HF treatment. For example, if a peptide methyl ester is synthesized by transesterification with methanol, the aspartic and glutamic methyl esters will not be cleaved by HF. Esters of peptides containing glutamic and aspartic acids can be synthesized if these latter residues are introduced as *t*-butyl esters, which are stable to transesterification and can be removed later by treatment with TFA.

Hydrazinolysis of peptide-resins yields blocked peptide hydrazides, which can be used for further synthesis by segment condensation methods. Diazotization of the hydrazide yields the azide for the coupling reaction. Here again, glutamic and aspartic acid residues will cause problems.

There has been some limited application of catalytic hydrogenolysis to cleavage of peptides from solid phase supports. Since hydrogenolysis reactions take place on the surface of a solid catalyst, this would at first glance appear to be incompatible with the solid phase system. If the peptide-resin is swelled in a solution of palladium salt, subsequent hydrogenation precipitates metallic palladium at the site of the ester bond which is then cleaved hydrogenolytically.

K. SIDE REACTIONS IN SOLID PHASE SYNTHESIS

Several undesirable side reactions which can take place in SPPS have been mentioned from time to time throughout this chapter. Certain of these will be considered here in greater detail.

1. Loss of Peptide from the Resin

As discussed earlier, the principal cause of loss of peptide from the resin is lack of adequate stability of the peptide-resin link to the conditions of synthesis. In the classical Merrifield SPPS system, as much as 1% of the peptide may be lost from the resin at each deprotection cycle. The Pam resin, in which the peptide-resin link is 100 times as stable as that in the classical system,

was introduced to overcome this problem. The necessary greater differential stability of the α-blocking group and the peptide-resin link can also be obtained by use of a much more labile α-protecting group. When TFA is used for deprotection, loss of peptide from the resin can also lead to chain termination by trifluoro-acetylation.

Another major source of loss of peptide from the resin is cyclization of dipeptides to diketopiperazines (see Figure 1-18). Since it was demonstrated that this reaction is catalyzed by weak acids, such as Boc-amino acids, the practice of reverse addition of Boc-amino acid and DCC during coupling of the third residue in a peptide chain was adopted.

2. Chain Termination

Termination of peptide chains by transfer of trifluoroacetyl groups from exposed hydroxyl groups on the resin[37] was discussed above (see Figure 1-17). This side reaction can be inhibited by use of more stable peptide-resin links and can be avoided by use of non-carboxylic acid reagents for removal of α-protecting groups. When trifluoroacetic or acetic acid remain in the resin at the coupling stage, they can be activated by DCC and thus terminate the peptide chain. It can also be avoided by use of symmetrical anhydrides or active esters for coupling reactions.

There have been some cases in which peptide chains failed to grow, even without apparent termination by trifluoroacetylation or other established mechanisms. Some evidence suggests that in certain sequences Boc groups may be totally resistant to deprotection reagents. In other cases, the amino group of the peptide may be completely buried in the polymer matrix in such a way as not to be accessible for coupling to activated amino acids. This type of difficulty has led to the use of alternate solvents for sucessive coupling reactions. For example, if monitoring shows that a coupling reaction has not proceeded to completion, if the initial coupling reaction was carried out in DCM the logical procedure is to use DMF for a recoupling reaction. Complete coupling can frequently be attained in this way. Interestingly, if the amino group is not available for coupling, it may also not be available for reaction with a monitoring reagent and thus a false negative monitoring test may lead to ultimate contamination of the product with short peptides or deletion sequences. Chain termination at glutamine residues is discussed below.

Recently a new mechanism for chain termination in SPPS has been found.[48a] In certain cases, commercial samples of Boc-amino acids were found to be contaminated with sec-butyloxycarbonyl derivatives. The s-Boc group is not removed by TFA, so chain growth ceases whenever such a group is added to the peptide. The group is cleaved by HF to yield short-chain by-products with free amino groups. This has led to the erroneous conclusion in some cases that Boc groups might be extremely and unpredictably stable in certain peptide sequences. While the latter may still be true, the discovery of this unanticipated contamination emphasizes the need for careful examination of purchased amino acid derivatives.

3. Deletion Peptides

If any given SPPS coupling reaction fails to go to completion, two N-terminal amino groups will then be present during the subsequent coupling reaction, one of these lacking the last residue coupled. In the next coupling step, both of these amino groups may be acylated, leading to propagation of the two chains, one of the chains lacking one amino acid residue. This often happens when a sterically hindered amino acid fails to couple completely and the next residue (or some later residue) is a small, non-hindered amino acid which will couple more readily to the remaining amino groups. Such peptides lacking one or more of the desired residues in the sequence have been called "deletion peptides" or "deletion sequences." As larger and more complex peptides are being synthesized by the solid phase method, more examples of difficult coupling sequences are being found. Monitoring of coupling reactions is especially important for detection of these problems. When such an incomplete coupling reaction is found, the first step, as mentioned above, is to recouple the same

residue in a different solvent or by use of a different coupling reaction. For example, if a DCC coupling reaction in DCM was used initially, the DCC reaction may be repeated in DMF or the operator may switch to a symmetrical anhydride or HOBt active ester. If monitoring still shows incomplete coupling, the operator may try again to complete the coupling reaction or he may choose to terminate the chain by acetylation. The latter procedure is frequently very useful in synthesis of small to medium sized peptides, where the acetylated short chain by-product can be separated easily from the desired product. In the synthesis of long peptides, such acetylation, if repeated often, may lead to the situation where no further chains are left to grow and yield the desired product. In this case, much greater efforts should be devoted to attaining complete coupling at every stage. Moreover, when the peptide is large, separation of these unwanted side products may not be easily achieved. A deletion peptide lacking a single simple amino acid may be impossible to separate from a large peptide. Fortunately, most of the amino acids which fail to couple are large or trifunctional residues, but this is by no means always the case. We have encountered at least one case of extreme difficulty in coupling Boc-alanine to an N-terminal alanine peptide-resin. This surprising event was obviously dependent upon the particular sequence at hand.

Several investigators have used chain-terminating reagents which will impart some characteristic to the undesired by-product to facilitate separation of it from the main product. Several of these are discussed in the review of Barany and Merrifield.[1]

4. Problems with Individual Amino Acids

Most of these problems have been discussed earlier, and will be mentioned again here for completeness and convenience of reference.

Problems with arginine residues usually arise if nitroarginine is used in synthesis. Current availability of tosyl arginine eliminates these problems. As mentioned above, there is still need for a suitable blocking group for arginine which is more labile than the tosyl group.

If Boc-Lys(Z) is used in SPPS, the side-chain blocking group will be slowly lost with repetitive deprotection cycles. This can lead to formation of significant amounts of branched chain peptides, the branches being on the lysine side chain. Use of the recommended more stable halogenated Z groups will avoid this problem.

Histidine remains a major problem in SPPS. Both of the currently recommended histidine derivatives, Boc-His(Tos) and Boc-His(Dnp), leave something to be desired. The tosyl group is too labile, and a more stable group should be developed for imidazole protection. Many frustrating hours have been lost in this laboratory in attempts to persuade histidine-containing peptides to continue growing in the vicinity of histidine residues. Problems can be anticipated when two or more histidine residues are adjacent or near each other. It may be necessary to call into play the entire repertoire of available histidine derivatives and coupling reactions. Careful monitoring is essential in these cases.

The major problem with aspartic acid in SPPS occurs when this residue is followed in the peptide sequence by a glycine, alanine, or serine residue. In these cases, treatment of the blocked peptide-resin with HF or other acid cleavage reagent may lead to a significant amount of succinimide peptide **(59)** in the crude product (see Figure 1-21). When the peptide is treated with water or dilute base the succinimide ring will be opened, but unfortunately the major product is usually not the desired normal aspartic peptide **(60)** but rather the undesired β-aspartyl peptide. **(61)**.In small peptides, some information on relative amounts of α- and β-aspartyl peptides can be obtained by electrophoresis at pH 2.8 (1 *M* acetic acid); the two peptides will have a different mobility due to the difference in pK of the free α-and β-carboxyl groups (α-pK = 2.5, β-pK = 3.5). The amount of β-aspartyl peptide formed is apparently sequence-dependent. Moreover, this electrophoretic method cannot be expected to differentiate between the two forms in long peptides.

Succinimide formation is much less of a problem if the aspartic residue is not esterified. This knowledge has led to development of

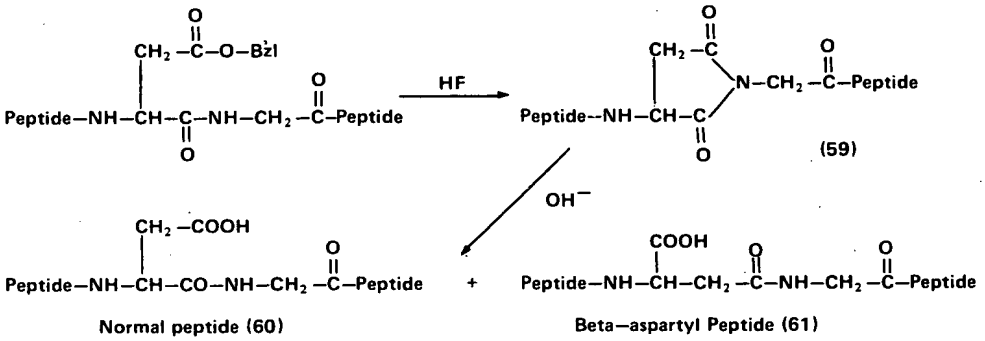


FIGURE 1-21.
Formation of *beta*-Aspartyl Peptides.

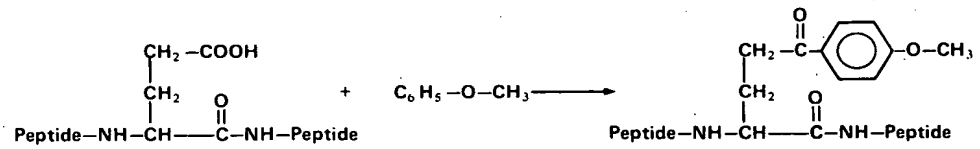


FIGURE 1-22.
Acylation of Anisole by Glutamyl Residues.

several different approaches for solving the succinimide problem. Perhaps the most practical approach is the use of a sterically hindered ester for the β-carboxyl of aspartic acid. In classical SPPS, this can be achieved by use of the cyclohexyl ester, and in labile SPPS, by use of the *t*-butyl ester. Neither of these branched-chain esters is subject to ready aminolysis by the adjacent peptide bond. Succinimide formation is prevented by use of the new "low HF" procedure for cleavage (see section J, above).

The major side reaction with glutamic acid residues in SPPS is acylation of the scavenger during HF cleavage; this Friedel-Crafts reaction is shown with anisole in Figure 1-22. This reaction takes place only after the protecting ester has been cleaved by the acidolytic reagent. HBr cleavage apparently causes less acylation than HF, while TFMSA can be expected to be worse in this respect. The reaction appears to be sharply temperature dependent, and this is a major reason for not carrying out HF cleavage reactions at temperatures above 0°. Acylation by glutamyl residues does not appear to be a problem in the labile system of SPPS where TFA is used for final cleavage. The acylation reaction is irreversible, and the undesired by-product must be removed from the desired peptide. Since the by-product contains an additional aromatic residue, it can usually be removed by partition methods such as ccd or partition chromatography. Acylation of the scavenger by glutamic side chains is prevented by use of the new "low HF" cleavage procedure (see section J, above).

Dehydration of asparagine and glutamine side chains to nitriles was discussed in section E, above. Both HOBt-assisted coupling reactions and active esters have been used to avoid this dehydration. On the other hand, the amide may be blocked by a group such as the xanthyl group, which also has its problems, as discussed above. Do not use symmetrical anhydrides for coupling asparagine and glutamine; they cause dehydration.

Cyclization of N-terminal glutamine residues to pyroglutamyl residues is a frequent cause of chain termination in SPPS.[50] Like ring closure

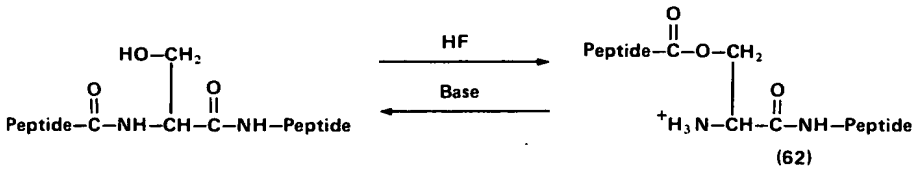


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FIGURE 1-23.
N-to-O Acyl Shift in Serine Peptides.

of dipeptide-resins to diketopiperazines, this reaction is catalyzed by weak acids, such as Boc-amino acids. For this reason, the inverse order of addition of coupling reagents should be used when DCC-mediated couplings follow glutamine residues in the synthesis. TFA appears to qualify as a catalytic weak acid in this case, especially at the low concentrations used for deprotection in labile SPPS systems. Ring closure will also be facilitated by use of polar aprotic solvents such as DMF. Use of a stable blocking group on the side-chain amide should also prevent cyclization.

Hydrolysis of glutamine and asparagine side-chain amides to acids can also be a problem in SPPS, particularly when these residues are adjacent or near to arginine or lysine residues.[49] This deamidation is markedly sequence-dependent; one sequence was found in which the amide had a half-life at room temperature and neutral pH of about 5 days. Such sequences present a serious problem for purification of synthetic peptides, especially in SPPS where extensive purification procedures may be necessary. The rate of hydrolysis is accelerated by acidic or basic solutions and by elevated temperatures, and has even been reported to occur during HF cleavage reactions. This deamidation can best be detected in synthetic peptides by electrophoresis at neutral pH. Purification procedures must be rapid, must avoid any pH extremes, and if possible should be carried out in systems having a low concentration of water. Preparative hplc should be ideal for such peptides.

The main problem to be anticipated with serine and threonine residues is the slow intramolecular migration of the acyl group from the α-amino group to the hydroxyl group (see Figure 1-23). This reaction is promoted by anhydrous strong acids such as HF and HBr. Very little migration can be anticipated in most peptides in the length of time during which the peptide is exposed to strong acid during cleavage of the peptide from the resin. Moreover, the migration is reversible by treatment of the O-acyl peptide with aqueous base. This reaction occurs with both serine and threonine, and the extent to which it occurs and the rate of migration will depend upon the amino acid sequence about the susceptible residues. In many cases, it will not be necessary to make the aqueous solution of the peptide alkaline in order to promote the reverse migration; in at least one case that has been studied (insulin) the reverse migration occurred rapidly at any pH above 3.5. Some chemists who wish to assure reverse migration have allowed the peptide to stand in dilute aqueous bicarbonate or ammonia solution at 0° for periods of up to one hour, but this is probably not necessary.

The principal difficulty with tyrosine is alkylation of the aromatic ring at the very reactive positions adjacent to the hydroxyl group. Attack on this position by alkylating and acylating ions produced during acidolytic cleavage reactions can be avoided by use of adequate amounts of effective scavengers. When O-benzyl tyrosine is used, attack can also occur by intramolecular migration of the benzyl group from oxygen to the 3-position of the ring (see Figure 1-15). This is a well-known reaction in organic chemistry, and is promoted by Friedel-Crafts catalysts. This alkylation can be markedly reduced by use of the 2,6-dichlorobenzyl ether and apparently does not occur if the hydroxyl group is protected as the 2-bromobenzyloxycarbonyl derivative; both of these are commercially available. The cyclohexyl ether has been recommended as being probably ideal for this purpose,

but is not commercially available at the present time. By-product peptides containing 3-alkyltyrosine residues can be separated from the desired product by partition methods, especially if the peptide is small. For example, the 3-benzyltyrosine derivative of angiotensin II or enkephalin has a much higher partition coefficient in ccd than the desired peptide, and is easily separated. The presence of a scavenger such as indole in deprotection reagents will prevent *t*-butylation of tyrosine residues. Alkylation of tyrosine during cleavage of the peptide from the resin is prevented by use of the new "low HF" procedure for cleavage (see section J, above).

Halogenation of tyrosine residues occurs very readily, and particular pains must be taken to avoid the presence of any free halogen or conditions which can generate free halogen throughout the procedures of SPPS. When HBr is used for cleavage, the gas must be scrubbed to remove bromine by passing it through a solution of anisole or resorcinol. Chlorination of tyrosine can occur during hydrolysis of peptides with HCl; addition of phenol to the hydrolysis mixture prevents this problem by trapping any chlorine present.

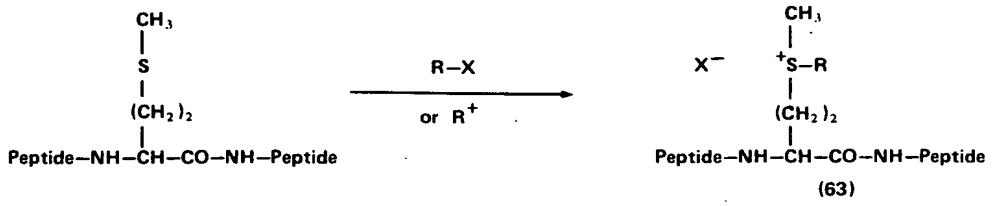
Problems associated with use of tryptophan in SPPS were discussed in section E.3.m, above. We would emphasize again that a blocking group on the indole of tryptophan does not seem to be necessary if a scavenger is included in the deprotection reagent. Tryptophan is also largely destroyed in classical systems of acid hydrolysis of peptides for analytical purposes. The very simple modification of adding mercaptoethanol (or some other thiol compound) to the hydrolysis mixture and carefully eliminating all oxygen will allow essentially quantitative recovery of tryptophan from hydrolysates of many peptides. This hydrolysis procedure converts cystine to reduced and mixed disulfide forms, thus interfering with normal quantitation of this residue as cystine. Cysteine emerges under proline in classical amino acid chromatograms. This is not a serious problem, since cysteine quantitation in hydrolysates is usually very poor; accurate quantitation of cysteine is obtained only by oxidation of it to cysteic acid before hydrolysis. Even though thiols will protect tryptophan from oxidation during acid hydrolysis, the operator should remember that degradation of tryptophan will begin as soon as the hydrolysate is exposed to air; rapid application of the sample to the amino acid analyzer is mandatory.

Problems associated with synthesis, purification and oxidation of cysteine-containing peptides were discussed above in section E.3.n. As mentioned in the preceding paragraph, analysis of cysteine in peptides by acid hydrolysis usually gives poor quantitation, and the peptide must be first oxidized with performic acid. This oxidation converts cysteine and cystine to cysteic acid; it also oxidizes methionine to methionine sulfone and usually destroys tryptophan. If HBr is used to destroy excess performic acid at the end of the oxidation, tyrosine will be destroyed by the bromine formed in this reaction.

Problems with spontaneous oxidation of methionine were discussed above in section E. If the spontaneous oxidation has occurred during the synthetic operations or if methionine sulfoxide was intentionally introduced into the peptide, inclusion of 1% 2-mercaptopyridine in the HF-anisole cleavage mixture has been reported[51] to effect reduction to methionine. If oxidation has occurred during purification (for example during ccd, where the solution is exposed to air throughout the process), methionine sulfoxide can be reduced to methionine in many cases by treatment overnight with 2-mercaptoethanol; N-methyl mercaptoacetamide has been reported[52] to be a superior reagent for this purpose. In some cases, rapid reduction can be effected by treatment at pH 7 with sodium borohydride; this does introduce salt into the peptide. Methionine sulfoxide residues in peptides are effectively reduced to methionine residues by use of the new "low HF" cleavage procedure (see section J, above).

Peptides containing methionine sulfoxide are more polar than the corresponding methionine peptides and can often be separated by partition methods; on reversed-phase hplc the sulfoxide peptide emerges ahead of the methionine peptide.

Oxidation of methionine to the sulfone is more difficult, and does not generally occur spontaneously. The sulfone is quite stable, and reduction of methionine sulfone is not practical.



(63)

FIGURE 1-24.
Alkylation of Methionine Residues to Form Sulfonium Peptides.

As was noted in the previous section, performic acid oxidation of peptides converts methionine to the sulfone; this oxidation may be useful for precise quantitation of methionine in hydrolysates, since it customarily emerges from amino acid analyzers very close to the buffer change artifact.

The sulfur of methionine is alkylated readily by alkyl halides or alkyl carbonium ions (such as are formed during acidolytic cleavage) (see Figure 1-24). The unintentional alkylation of methionine by chloromethyl groups on the resin during attachment of methionine in the initial step of SPPS was mentioned above. If a sufficient concentration of active nucleophilic scavengers is not present during acidolytic cleavage of peptides from the resin, methionine may be alkylated. For example, in a synthesis of methionine-enkephalin where O-benzyltyrosine was used, two undesired side-products were separated from the desired peptide by ccd. The by-product in which methionine had been converted to the S-benzyl sulfonium derivative was much more polar **(63)** than Met-enkephalin and had a lower partition coefficient. On the other hand, the peptide containing 3-benzyltyrosine was more hydrophobic and had a higher partition coefficient. Both by-products were readily separated from the desired peptide. Alkylation of methionine during cleavage of the peptide from the resin is prevented by use of the new "low HF" procedure for cleavage (see section J, above)

While many proline-containing peptides have been synthesized by SPPS with no difficulty, in some sequences proline may be the cause of significant chain termination, usually due to trifluoroacetylation. The Tfa groups may be present in the resin due to inadequate neutralization or may be transferred from exposed hydroxyl groups on the resin, as discussed earlier. The unusual basicity of proline (the pK is about one unit higher than with other amino acids) and the presence of the ring structure, which evidently holds the amino group in an exposed position, both favor its participation in nucleophilic reactions. These problems were discussed above. Difficulties in monitoring SPPS reactions of N-terminal proline peptides were discussed in section I, above. Loss of dipeptides from the resin by cyclization to diketopiperazines was discussed above, and is particularly bad with proline-containing dipeptides. There are also cases where a peptide with a terminal proline group has cyclized spontaneously in solution with cleavage of the peptide chain.

Slow coupling reactions may be anticipated in SPPS when either the entering Boc-amino acid or the N-terminal residue on the peptide-resin is sterically hindered. Among naturally occurring amino acids, those with β-branching give problems: valine, isoleucine, threonine. Among unnatural amino acids, N-alkyl or α-alkyl amino acids are particularly bad in this respect. With these residues, coupling reactions may need to be prolonged for many hours and repeated in order to effect complete coupling.

5. Racemization

The fortunate general lack of racemization in SPPS when single α-urethane protected amino acids are coupled has been discussed above. While these derivatives do not generally form azlactones **(55)**, the usual intermediate which promotes racemization, if an activated derivative is kept for extended periods in a polar aprotic solvent such as DMF some racemization can occur. This is another argument for repeti-

tion of coupling reactions to attain completion rather than undue prolongation of the initial coupling. It has recently been shown[53] that *p*-dimethylaminopyridine promotes racemization when it is used to catalyze formation of ester or peptide bonds. Since DCC-catalyzed formation of ester bonds may be slow, the usual procedure when attaching a Boc-amino acid to a hydroxymethyl resin involves addition of DMAP to accelerate the esterification. DMAP has also been found to accelerate the rate of slow peptide-forming reactions. In both these cases there is danger of racemization. When DMAP is used, one should use only a catalytic amount, rather than a full equivalent as has been formerly recommended. If amino acids blocked with protecting groups other than urethanes are used or if blocked peptides are coupled in SPPS, racemization can be expected to be much more of a problem, and adequate precautions must be taken.

L. PURIFICATION OF PEPTIDES

1. Methods

Methods for a variety of peptide purification procedures are given in chapter 2 of this book. The procedures described have been found to be very useful in purification of peptides synthesized by SPPS. Countercurrent distribution and partition chromatography have been particularly useful for purification of small peptides, unless the peptide is extremely hydrophilic. Many peptides synthesized by SPPS can be obtained in homogeneous condition after a single ccd run of 100-200 transfers if the peptide contains up to 10 or 15 amino acids. For larger peptides, serial application of several different methods will probably be necessary. Molecular sizing on gel columns is a useful first step in this case; this will provide a fraction of approximately the correct molecular weight. One must be aware that abnormal results can be obtained due to peptide-peptide and peptide-gel interactions which cause peptides to elute in positions not characteristic of their true molecular weight. These aberrations can frequently be overcome by use of strong acetic acid solutions or eluants containing pyridine. More rapid purification can be anticipated when successive procedures utilize different separation criteria, rather than mere repetition of the same type of procedure. For example, if a procedure based on partition (such as ccd or partition chromatography) does not yield a pure peptide, the next procedure might well be one where the separation is made on the basis of charge, such as ion-exchange chromatography or preparative electrophoresis. Preparative hplc can be expected to play an ever increasing role in purification of synthetic peptides. A simple, inexpensive instrument for continuous flow preparative electrophoresis of peptides would be a great contribution to the peptide laboratory. Unfortunately, such an instrument is not available at the present time.

When large peptides are synthesized, extraordinary measures are generally required for adequate purification. Hplc may be particularly useful in these cases. Affinity chromatography, using either antibodies specific to the peptide being synthesized or those exploiting some other characteristic of the desired product, may be particularly useful, and even necessary.

2. Assessment of Purity

It cannot be stressed too much that chemists must not rely upon a single method or a single class of methods for ascertaining homogeneity of synthetic peptides. For example, both tlc and electrophoresis should be used, since they examine different properties of the peptide. Hplc has extremely high resolving power, and is very useful for determining homogeneity of products. It must be emphasized that a single isocratic run (elution with a single solvent) cannot assure homogeneity of the product, even if a single peak is obtained. There may be other components of the injected material which adsorb very strongly to the stationary phase and are not seen under these circumstances. Both isocratic and gradient runs should be used. A steep gradient will cover a wide range of peptide properties and should allow most contaminants to be seen. The isocratic run will then provide the highest resolution to ascertain presence of materials very similar to the main product. The eluant for the

isocratic run should be chosen so that the peptide has a moderate retention time on the column; if it emerges too fast, it can obviously not be separated from fast-moving contaminants. Separate isocratic runs should be made in which a normal amount and an overload amount of peptide are injected; the overloaded run will provide additional sensitivity for detection of impurities.

Determination of the amino acid sequence of the synthetic peptide is a very sophisticated way to estimate homogeneity of the final product. This is particularly useful with large peptides, and is good for detection of deletion sequences in the product. If a fraction of the product is missing any given amino acid residue, the phenylthiohydantoin from that step of the Edman degradation will show not only the expected residue but some of the residue which follows it in the sequence. The amount of this "preview" is a measure of the fraction of the product which lacks the amino acid in question. After many steps, the Edman degradation becomes very "noisy" due to incomplete reactions, and the yield at each step decreases, but it can frequently be used effectively to assess the homogeneity of a peptide through as many as 40-60 residues. The Edman degradation is useful not only in this way for determination of the homogeneity of the finished pure peptide, but also can be applied to the peptide-resin directly from the solid phase synthesis in order to obtain a quantitative measure of the progress of the coupling. Deletion sequences can be identified in this way directly. Hplc systems have been developed for identification of the phenylthiohydantoin derivatives of the blocked amino acids as they are cleaved from the peptide-resin. A solid phase sequencer, such as the Sequemat®, is very practical for this purpose, or peptide-resin beads can be added to the cup of a spinning cup sequencer.

Accurate amino acid analysis of a hydrolysate of a final pure peptide is an absolute requirement. Elemental analysis, the long-time standby of the organic chemist, is not very useful in peptide work, since the percent composition does not vary widely among peptides. While amino acid analysis does not assure that the synthetic peptide has the right sequence, it does provide information on the composition of the product. Pure peptides should give the expected ratios for all amino acids except those which are destroyed partially in hydrolysis. These problems are discussed above in section K. Since many carefully lyophilized peptides will still contain very considerable quantities of solvent, the amino acid analysis, if carefully performed, will also give an accurate working molecular weight of the product. This latter value is essential when one wishes to prepare a solution of the peptide having a known molar concentration. Since some homogeneous peptides may be found to contain as much as 40% solvent or other inert material, significant errors can be introduced unless the true working molecular weight is used. Unfortunately, there is also ambiguity about what different individuals mean by the terms "peptide content" and "purity." This arises in part because of different practices in calculation of molecular weight. For example, since most peptides will be either acidic or basic and will normally contain a counter ion (frequently acetate or trifluoroacetate), different theoretical molecular weights will be obtained if one calculates the molecular weight of actual polymerized amino acids or if one includes the salt which is properly considered a part of the molecular weight. The sum of the residue weights of the constituent amino acids in a peptide gives what we call the "bare bones" molecular weight. This value, divided by the actual working molecular weight found by amino acid analysis gives the "peptide content." If one adds to the "bare bones" molecular weight the weight of acid (or base) molecules necessary to yield a neutral molecule, one obtains the theoretical molecular weight. The theoretical molecular weight divided by the working molecular weight gives a value for the purity of the product when expressed as a percent. When transmitting this information to individuals who will use the synthetic peptide, it is important to stress that this "purity" is not a matter of lack of homogeneity of the synthetic peptide but rather is an expression of the necessary salt and associated solvent in the final form of the material. In this laboratory, all these parameters, as well as the ratios of the amino acids in the hydrolysate, are calculated rapidly

and reported effectively by a VisiCalc® program on a microcomputer.

M. PROBLEMS IN HANDLING AND USE OF PEPTIDES

Because of the special properties of peptides, their storage, handling and use are beset by many difficulties. Peptide problems can be physical, chemical or biological in nature. All those persons synthesizing and using peptides should be aware of these difficulties.

Although many peptides are quite stable when stored dry, even at room temperature, in general one should take every precaution to keep pure peptides cold and dry. The large amount of solvent remaining in some "dry" peptides after lyophilization can slowly attack the peptide upon standing. Particularly if glacial acetic acid has been used as a final lyophilization solvent, traces of remaining acidity may cause hydrolysis of labile amide bonds in glutamine and asparagine residues and at peptide C-termini. In addition, the known acid lability of aspartyl residues in peptides can lead to cleavage of the peptide chain at aspartic residues. Diketopiperazine formation from dipeptide esters is very rapid, and diketopiperazine formation with chain cleavage has even been observed in some larger peptides. N-terminal glutamine residues are particularly labile, and cyclize to the pyroglutamyl peptide. The basicity of arginine and lysine residues can aggravate some of these problems, and the catalytic ability of the imidazole ring of histidine is well known.

When containers of dry peptides are removed from the cold, they must be allowed to warm to room temperature completely before the container is opened. Otherwise, atmospheric moisture will condense on the peptide and proceed to hydrolyze it upon return to storage. Even if the peptide should be stable to the added moisture, the increased weight would lead to error in future attempts to make solutions of known concentration.

These degradative reactions proceed much more rapidly once the peptide is dissolved in water. Particularly if the solution is not neutral, problems may arise with sensitive peptides. Glass surfaces are inherently basic, due to the alkali in the glass; old glass is particularly bad. Peptide solutions stored in glass can thus decompose due to alkaline hydrolysis if buffers are not present. All containers which will be used for storage of peptides and for making peptide solutions should be thoroughly washed and finally rinsed with acetic acid solutions before careful drying. Silanization will stabilize the glass surface and help prevent some of these problems. The surface of glass is negatively charged, and basic peptides will adsorb avidly to its surface. Hydrophobic peptides also will adsorb by nonpolar forces. This is a major problem when biologically active peptides are being used, since most of them are basic and they are generally used in extremely dilute solutions. All glassware for peptide solutions should be acid washed and silanized. Surface adsorption of peptides is not so serious with polyethylene and polypropylene. On the other hand, polystyrene and acetate plastics adsorb peptides extremely avidly. Their ability to adsorb peptides and proteins is exploited in solid phase immunoassay procedures. A popular procedure for making dilute solutions of biologically active peptides is to include 0.01 *M* acetic acid in the medium; this helps prevent adsorption of the peptide to glass and plastic surfaces. In one set of experiments, a peptide (substance P) showed a 1000-fold increase in potency when the solution was acidic.

It has long been the practice of immunochemists to prevent adsorption of very dilute solutions of radioactive tracer peptides to glass by including protein in the solution; such additives as 0.1 mg/mL of bovine serum albumin or 1% plasma have been used. In experiments on membrane receptors for peptide hormones the membrane particles carrying the bound tracer are frequently separated from the solution by filtration through glass fiber filters. In the case of basic peptides such as bradykinin and substance P, much peptide from solution adsorbs to the filters and confuses the result. Such adsorption has been minimized by prewashing the filters with a 0.1% solution of polyethyleneimine. Use of such preventive measures may help produce clearer experimental results, although in many cases such additives are not acceptable.

Some hydrophobic peptides may be difficult to dissolve. In some cases they may be significantly easier to dissolve in pure water than in physiological saline solutions. Depending upon the use of the solution, water-insoluble peptides may be dissolved first in such solvents as DMSO, propylene glycol, acetic acid or aqueous methanol, and then diluted with water. Such peptides dissolve much more readily if they were lyophilized from a fairly dilute solution so that they are light and fluffy. In contrast, peptides dried by evaporation or lyophilized from very concentrated solutions may be in the form of dense, large particles, which are very difficult to dissolve.

Oxidation is a serious problem with methionine-containing peptides. It is particularly rapid when the peptide is in a very dilute solution or adsorbed to a surface. Stock solutions of labile peptides should be kept fairly concentrated and should be divided into small aliquots for freezing. Repetitive freezing and thawing of solutions should not be allowed. Once the peptide solution is diluted for biological experiments, the solutions should be kept cold and used as soon as possible. Previous bubbling of the solvent with nitrogen may help to retard oxidation. Surface-catalyzed oxidation of methionine peptides can be quite rapid during tlc. If a two-dimension tlc of methionine-enkephalin is done, significant oxidation takes place when the plate is dried between first and second runs.

Cystine-containing peptides may undergo rapid disulfide interchange when they are stored in solution at neutral or alkaline pH. Solutions of oxytocin and vasopressin, for example, are rapidly inactivated by such treatment, and are reported to be most stable when slightly acid (pH 5) and just above freezing; freezing is reported to promote degradation. The disulfide interchange that occurs with these peptides leads to formation of biologically inactive dimers and polymers.

Chemists are not usually concerned with bacterial contamination of their materials, but they should remember that bacteria and fungi are omnipresent, and aqueous solutions of peptides make good food for them. Colonies of microorganisms have become established in Sephadex® columns and subsequently degraded all peptides passed through the column. The standard practice of including sodium azide in the solution with which gel columns are stored must not be overlooked.

Careful attention to these points will save much grief and help to avoid contamination of the literature with inaccurate peptide information.

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